U. S. TREASURY DEPARTMENT
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STUDIES ON OXIDATION-REDUCTION

XVI. THE OXAZINES: NILE BLUE, BRILLIANT CRESYL BLUE, METHYL CAPRI BLUE, AND ETHYL CAPRI BLUE

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SUPPLEMENT No. 92
TO THE
PUBLIC HEALTH REPORTS

UNITED STATES
GOVERNMENT PRINTING OFFICE
WASHINGTON : 1931

For sale by the Superintendent of Documents, Washington, D. C. - - - - - - Price 15 cents

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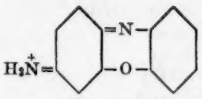
STUDIES ON OXIDATION-REDUCTION

XVI. THE OXAZINES: NILE BLUE, BRILLIANT CRESYL BLUE, METHYL CAPRI BLUE, AND ETHYL CAPRI BLUE[1]

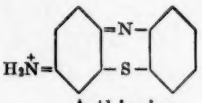
Introduction

In 1879 Meldola obtained a new dyestuff by heating equimolecular quantities of β-naphthol and nitrosodimethylaniline hydrochloride in glacial acetic acid. This and similar compounds were called Meldola dyes. Since the elucidation of their chemical structure by the work of Möhlau and of Kehrmann, they have come to be known as the oxazine group of dyes.

Structurally, these compounds are analogous to the thiazines, an oxygen atom replacing the sulphur in the latter,



An oxazine



A thiazine

and they have many properties in common.

As a class the oxazine dyes are of interest both chemically and biologically. They are brilliant dyes capable of reversible oxidation-reduction and may, therefore, furnish useful indicators. Certain of them are employed in various methods of staining live and dead tissue and in other biochemical studies; and several have been rather extensively used in recent years in the study of the permeability of cell membranes. A clearer understanding of some of the fundamental physico-chemical properties of these compounds should be valuable not only in the interpretation of the results obtained from their use as indicators or stains but also in the design of new applications.

This study is a continuation of the systematic examination of the various dye classes; and the main interest concerns the position of the oxidation-reduction equilibrium potentials of a simple oxazine analogous to methylene blue. With this end in view, tetramethyl Capri blue (*methyl Capri blue*) was synthesized and studied. The tetraethyl homologue (*ethyl Capri blue*) was also prepared and measured to obtain additional information on the effects of substitution on dissociations and equilibrium potentials.

[1] Submitted for publication Oct. 1, 1930. Completing details in connection with this study were made in the Laboratory of Physiological Chemistry, Johns Hopkins University School of Medicine, Baltimore, Md.

Nile blue and brilliant cresyl blue are oxazines both of which enjoy a certain vogue in histological and biochemical practice. It seemed desirable, therefore, to know their constants,[2] and so these systems were also included in our study. With these dyes, some disconcerting and confusing difficulties were encountered which disclosed certain rather important and interesting phenomena. The colloidal behavior of Nile blue solutions introduced marked concentration effects in the electrode measurements. The pronounced instability of brilliant cresyl blue in water served to emphasize an apparently general property of the oxazines and perhaps of analogous classes of compounds. The difficulties and sources of error encountered by Clark, Cohen, and Gibbs (*cf.* Paper VIII of this series) in the study of the methylene blue system were more or less accentuated in the case of these oxazines.

Nevertheless, general outlines of the four oxazine systems have been established with a fair degree of precision. On the scale of electrode potentials, the methyl Capri blue system is more electronegative than the corresponding thiazine, methylene blue. The ethyl Capri blue system is still more electronegative from pH 7 to 10. At pH 7 it occupies a position between indigo tetrasulphonate and indigo trisulphonate. (See fig. 3 and Table 36.)

This position of the ethyl Capri blue system is of more than ordinary importance in relation to studies of the intensity of intracellular oxidation-reductions, for Chambers, Pollack, and Cohen (1929) report that this dye is apparently partly reduced in the cytoplasm of certain marine ova while the more electropositive indigo tetrasulphonate is unaffected owing, presumably, to its toxicity.

Until better compounds are found, these dyes should be serviceable as basic oxidation-reduction indicators where the acid indigo sulphonates prove unsatisfactory.

In the following presentation of the experimental studies each of the four compounds will be discussed in a separate section. Nile blue and brilliant cresyl blue will be discussed first because they illustrate to an extreme degree the peculiarities that must be well understood for intelligent use of the compounds.

Experimental Procedure

The electrode potential measurements were carried out at 30° C. ±0.02 with the equipment previously described (Collected Reprints, 1928). The ultimate working standard was the potential of the hydrogen electrode in M/20 potassium acid phthalate, to which was assigned the value −0.2386 volt at 30° C. The reference electrode was the saturated KCl–calomel cell, which was frequently checked

[2] Rapkine, Struyk, and Wurmser (1929) and Vellinger (1929) have reported a limited series of measurements. See Appendix.

against the above standard. Liquid junctions were made with saturated KCl solution and the liquid junction potentials were ignored.

By means of the customary methods, the E'_0 value of each oxazine system was determined at one or several pH values by titration of the oxidant or the reductant in buffer solution. Next, a fixed mixture of reductant and oxidant was carried through a wide range of pH to determine the variation of E'_0 with pH, from which the E'_0: pH curve was constructed. The characteristic bends in the curve yielded the values for the several dissociation constants of each system. The reader is referred to previous papers in this series for a more detailed account of the experimental procedure.

BUFFER SOLUTIONS

The composition of each of the buffer solutions is recorded in Table 1. In the construction of these solutions the purpose was to have the entire series of uniform concentration (0.1 molar) with respect to sodium. The last column in the table gives the computed approximate ionic strengths.

No unusual care was taken in the exact reproduction of these solutions from time to time, because hydrogen electrode measurements were taken shortly before or after the use of any buffer.

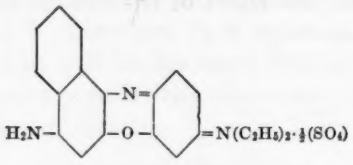
In subsequent discussions these buffers will be referred to by number; solutions numbered ½ (except No. 24½) refer to buffers made by mixing equal volumes of solutions having adjacent numbers.

For the various electrode measurements and titrations, except where stated otherwise, these buffers were diluted in the proportion of 50 c. c. buffer to 5 c. c. dye solution. Hydrogen electrode determinations were made on the buffers similarly diluted with water, and these values were considered to be the same for the corresponding buffered dye solutions, the effect of the minute content of dye being regarded as negligible.

TABLE 1.—*Composition of buffer solutions*

Solution No.	Composition	Approximate ionic strength
1S	250 c. c. M/10 Na_2SO_4+ 250 c. c. M/10 H_2SO_4+ 0 c. c. water	0.30
2S	250 c. c. M/10 Na_2SO_4+125 c. c. M/10 H_2SO_4+125 c. c. water	.23
3S	250 c. c. M/10 Na_2SO_4+30 c. c. M/10 H_2SO_4+220 c. c. water	.17
1A	100 c. c. M/1 NaOH+200 c. c. M/1 CH_3COOH+700 c. c. water	.10
2A	100 c. c. M/1 NaOH+125 c. c. M/1 CH_3COOH+775 c. c. water	.10
3A	100 c. c. M/1 NaOH+115 c. c. M/1 CH_3COOH+785 c. c. water	.10
1	250 c. c. M/5 NaCl+250 c. c. M/5 HCl+000 c. c. water	.20
2	250 c. c. M/5 NaCl+125 c. c. M/5 HCl+125 c. c. water	.15
3	250 c. c. M/5 NaCl+30 c. c. M/5 HCl+220 c. c. water	.11
4	250 c. c. M/5 citric acid+50 c. c. M/5 NaOH+450 c. c. M/5 NaCl+250 c. c. water	.10
5	250 c. c. M/5 citric acid+125 c. c. M/5 NaOH+375 c. c. M/5 NaCl+250 c. c. water	.10
6	250 c. c. M/5 citric acid+210 c. c. M/5 NaOH+290 c. c. M/5 NaCl+250 c. c. water	.11
7	250 c. c. M/5 citric acid+300 c. c. M/5 NaOH+200 c. c. M/5 NaCl+250 c. c. water	.14
8	250 c. c. M/5 citric acid+400 c. c. M/5 NaOH+100 c. c. M/5 NaCl+250 c. c. water	.18
9	250 c. c. M/5 citric acid+500 c. c. M/5 NaOH+0 c. c. M/5 NaCl+250 c. c. water	.23
10	208 c. c. M/5 citric acid+500 c. c. M/5 NaOH+0 c. c. M/5 NaCl+292 c. c. water	.28
11	185 c. c. M/5 citric acid+500 c. c. M/5 NaOH+0 c. c. M/5 NaCl+315 c. c. water	.31
12	250 c. c. M/5 Na_2HPO_4+230 c. c. M/5 HCl+520 c. c. water	.10
13	250 c. c. M/5 Na_2HPO_4+190 c. c. M/5 HCl+560 c. c. water	.11
14	250 c. c. M/5 Na_2HPO_4+145 c. c. M/5 HCl+605 c. c. water	.12
15	250 c. c. M/5 Na_2HPO_4+100 c. c. M/5 HCl+650 c. c. water	.13
16	250 c. c. M/5 Na_2HPO_4+40 c. c. M/5 HCl+710 c. c. water	.14
17	250 c. c. M/5 Na_2HPO_4+15 c. c. M/5 HCl+735 c. c. water	.15
18	250 c. c. M/5 H_3BO_3+10 c. c. M/5 NaOH+490 c. c. M/5 NaCl+250 c. c. water	.10
19	250 c. c. M/5 H_3BO_3+16 c. c. M/5 NaOH+484 c. c. M/5 NaCl+250 c. c. water	.10
20	250 c. c. M/5 H_3BO_3+30 c. c. M/5 NaOH+470 c. c. M/5 NaCl+250 c. c. water	.10
21	250 c. c. M/5 H_3BO_3+55 c. c. M/5 NaOH+445 c. c. M/5 NaCl+250 c. c. water	.10
22	250 c. c. M/5 H_3BO_3+80 c. c. M/5 NaOH+420 c. c. M/5 NaCl+250 c. c. water	.10
23	250 c. c. M/5 H_3BO_3+160 c. c. M/5 NaOH+340 c. c. M/5 NaCl+250 c. c. water	.10
24	250 c. c. M/5 H_3BO_3+240 c. c. M/5 NaOH+260 c. c. M/5 NaCl+250 c. c. water	.10
24½	250 c. c. M/5 H_3BO_3+260 c. c. M/5 NaOH+240 c. c. M/5 NaCl+250 c. c. water	.10
25	125 c. c. M/5 Na_2HPO_4+40 c. c. M/5 NaOH+210 c. c. M/5 NaCl+625 c. c. water	.14
26	125 c. c. M/5 Na_2HPO_4+90 c. c. M/5 NaOH+160 c. c. M/5 NaCl+625 c. c. water	.17
27	125 c. c. M/5 Na_2HPO_4+150 c. c. M/5 NaOH+100 c. c. M/5 NaCl+625 c. c. water	.18
28	250 c. c. M/5 NaOH+250 c. c. M/5 NaCl+500 c. c. water	.10
29	250 c. c. M/5 NaOH+750 c. c. water	.05
30	250 c. c. M/5 NaOH+250 c. c. water	.10

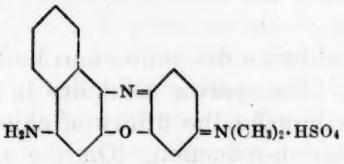
Ethyl Nile Blue



This compound is the commercial Nile blue A or diethyldiaminophenonaphthoxazine sulphate (hereinafter called Nile blue).

In 1888 Reissig disclosed in German patent No. 45268 that the reactive mononitroso compounds of alkyl derivatives of *m*-aminophenol condense with a number of primary, secondary, and tertiary aromatic amines to produce new dyes (oxazines). He described the

preparation of ethyl Nile blue sulphate; and for the dimethyl homologue he proposed the following structure:



In this connection, see the discussion on page 6.

The oxazines are formed in general by the action of nitrosodimethylaniline, nitrosophenols or the corresponding quinone chlorimides upon phenols, and also by the action of hydroxynitrosodimethylaniline upon amines. According to Möhlau and Uhlmann (1895), the union of two aromatic nuclei by a nitrogen and oxygen atom as in the oxazines produces a greater stability toward acids than is found in the quinazine dyes (indophenols and indamines).

SYNTHESIS, PURIFICATION, ANALYSIS, AND PROPERTIES

Preparation of Nile blue chloride.—The dye was made by condensing α-naphthylamine with nitroso-*m*-diethylaminophenol [3] in acetic acid solution. α-Naphthylamine hydrochloride, 100 gm., was suspended in a boiling mixture of 850 c. c. glacial acetic acid and 200 c. c. water. (The amine was not all dissolved.) To this boiling suspension was added slowly 100 gm. of nitroso-*m*-diethylaminophenol. In about 5 minutes a vigorous reaction occurred and the product crystallized as a thick paste. The reaction subsided in five minutes and the mixture was held in a boiling water bath for three hours. The product was a mass of bronzy green crystals. This was cooled to 40° and filtered with suction. It was then suspended in 1,500 c. c. distilled water, boiled a few minutes, and filtered once more. A strong amine odor was noticeable. After suspending the crystals in 1,000 c. c. warm water, adding 500 c. c. water containing 10 c. c. concentrated HCl, boiling the mixture and cooling, the odor was no longer detectable. The crystals were then filtered off, washed with water, and dried over stick NaOH.

Recrystallization of Nile blue chloride.—Ten grams of the crystals secured as above noted were suspended in 1,000 c. c. water brought to incipient boiling [4] and filtered hot. The process was repeated on the residue until all was dissolved and about 10 liters of solution obtained. Such a solution of ethyl Nile blue chloride does not crys tallize out spontaneously unless HCl or other chloride is added.

[3] The nitroso derivative of *m*-diethylaminophenol was obtained in 95 per cent yield by the Badische method (Ger. Pat. 45268; Möhlau, 1892).

[4] If the saturated Nile blue solution is boiled vigorously there occurs decomposition as evidenced by the appearance of an amine odor.

The introduction of 1 c. c. concentrated HCl per liter of solution caused a prompt crystallization of the product. After standing about 16 hours, it was filtered and dried over stick NaOH. The analyses are shown in Table 2.

The nitrogen and chlorine determinations both show purity in excess of 95 per cent. The sparing solubility in dilute buffers at ordinary temperatures impairs the utility of chloride of Nile blue as an indicator of oxidation-reduction. On the other hand, the low solubility and the ease of obtaining a product of apparent high purity make this compound admirably suited for investigations of specific salt effects, micelle formation, and phenomena associated with solutions approaching the colloidal state.

Purification of Nile blue sulphate.—The material selected for electrode measurements was a commercial product made by the National Aniline and Chemical Co., certified by the Commission on Standardization of Biological Stains (NNb1). The original sample, intended for fat-staining, was unsuitable for satisfactory measurements, because of oxazone present. However, after removal of the oxazone by extraction with ether a fairly satisfactory product (sample H) was obtained. The analyses are shown in Table 2, last two lines.

On the basis of nitrogen content, this sample shows 85.5 per cent purity, a value which was frequently confirmed by $TiCl_3$ titrations and was approached but never exceeded in other specimens of Nile blue sulphate that were examined.

All analyses for sulphur yielded values much in excess of the theoretical. The same thing was found in the Nile blue sulphates synthesized in our laboratory. An obvious conclusion would be that excess sulphuric acid is retained very firmly by this colloidal material and is not easily removed by the ordinary methods of washing and recrystallization. However, in our best specimen (sample H) the excess sulphur is almost exactly equivalent to *twice* the accepted theoretical amount of sulphate in the compound. Assuming this as a tentative basis for calculation we find that the discrepancy in nitrogen is also approximately accounted for. Thus we find our analyses of Nile blue sulphate to be consistent with $C_{20}H_{20}N_3O \cdot HSO_4$, the composition originally assigned by Reissig (see p. 5) and not with the dibasic salt $(C_{20}H_{20}N_3O)_2SO_4$ which is currently accepted. (See, however, Thorpe, 1907.)

Having in mind, however, the difficulties in preparing Nile blue sulphate and in its purification, we do not wish to insist on this interpretation without additional experimental support. It may be added that the electrode measurements to be described support the above analytical results to the extent that they do not reveal the presence of appreciable amounts of active impurities in sample H of Nile blue sulphate. Moreover, the relatively greater solubility in water of the

sulphate of Nile blue as compared to the chloride or nitrate is not readily explicable on the basis of its accepted structure as the dibasic salt.

A number of other products were examined, but they showed no superiority over sample H. Most of them yielded an ash on incineration (probably from Na_2SO_4). This is illustrated in Table 2 by sample A, a Badische product obtained from the Bureau of Standards (Textile Alliance Collection). Incidentally, if the sulphur analysis of this sample be corrected by deducting the amount corresponding to 3.8 per cent Na_2SO_4 (ash), the purity (72.0 per cent) on the basis of sulphur corresponds nearly to the purity (69.9 per cent) on the basis of nitrogen only if the formula Nile blue-HSO_4 is assumed.

TABLE 2.—*Analyses of Nile blue*

Name	Sample	Formula	Mol. wt.	Nitrogen		Purity on basis of N	Chlorine		Sulphur		Ash
				Found	Calc.		Found	Calc.	Found	Calc.	
				Per cent	*Per cent*	*Per cent*	*Per cent*	*Per cent*	*Per cent*	*Per cent*	*Per cent*
Ethyl Nile blue chloride	------	$C_{20}H_{20}N_3OCl$	353.7	11.37	11.88	95.7	9.68	10.02	------	------	0
Ethyl Nile blue sulphate	A	$C_{20}H_{20}N_3O \cdot 1/2SO_4$	366.3	7.07	11.47	61.6	0	0	6.42	4.38	3.8
----do	H	-----do	366.3	9.81	11.47	85.5	0	0	7.40	4.38	0
----do	H	$C_{20}H_{20}N_3O \cdot HSO_4$	415.3	9.81	10.12	96.9	0	0	7.40	7.72	0

Properties.—Ethyl Nile blue sulphate is the major constituent of Nile blue A of commerce. It dissolves to a small extent in cold water and is more soluble in hot. The chloride (also the nitrate) is much less soluble than the sulphate. When an aqueous solution of the dye is added to buffers, the resulting solution is blue from pH 1 to 8.4; a slight tint of fluorescent red-violet appears at about pH 9.4; the red becomes predominant at higher alkalinity and a red precipitate of the base appears. A quick and convenient means of distinguishing between ethyl Nile blue and the dimethyl homologue is the following: Addition of some 1 per cent solution of KOH to a solution of ethyl Nile blue produces a pink coloration, to methyl Nile blue a yellow coloration.

Nile blue reduced by $Na_2S_2O_4$ or by hydrogen in the presence of platinum black becomes colorless or straw yellow depending on concentration in buffers from pH 1 to 8.4 and reddish from pH 9.4 on. The reduced solutions are quickly reoxidized by air from pH 6.4 to 10; but the air oxidation is slow or absent in acid solutions from pH 1 to 5.4. Reoxidation by ferricyanide is practically instantaneous throughout the above range of pH.

The reductant of Nile blue may be obtained as white needles easily soluble in water.

ELECTROMETRIC STUDIES

The following electrometric analysis of the Nile blue system is based upon two samples of the diethyl derivative: (1) The chloride synthesized in the laboratory, and (2) the sulphate (sample H), a purified commercial product. Numerous titrations were made of various other specimens but to avoid confusion the results are omitted here. It should be stated, however, that evidences were found of the presence of considerable amounts of active impurities in commercial samples of the compound, impurities which interfere with the precise outline of Nile blue as an oxidation-reduction system but which, e. g., Nile blue oxazone, are intentionally present to meet certain specific requirements for biological staining or for dyeing fabrics.

Before proceeding to the oxidation-reduction measurements, it is necessary to discuss briefly some factors that influence the electrode measurements.

EFFECT OF LIGHT ON LEUCO-NILE BLUE

It will be recalled (Paper VIII) that leuco-methylene blue when exposed to light from the near ultraviolet portion of the spectrum becomes reoxidized to methylene blue, which betrays its presence by the increasing blue coloration of the originally water-white solution. It was suspected that Nile blue might be similarly light-sensitive. Therefore the following test was made: A carbon arc operated on a 110-volt A. C. current was placed 30 cm. from a nitrogen-protected soft glass (2 mm. wall) reservoir containing 0.000275 molar aqueous leuco-Nile blue chloride. The solution was a clear yellow at the start, and after an irradiation of over one hour no color change was perceptible. Under similar conditions leuco-methylene blue would have colored up within a few minutes.

The negative outcome to this experiment is opposed, however, by the observation that electrometric titrations of the above reduced solution with benzoquinone indicated the presence of about 5 per cent oxidant at the start. It was also observed that about 10 per cent oxidant must be present in the reductant to be perceptible to the eye. The matter was not pursued further.

EFFECT OF VARIATIONS IN CONCENTRATION OF OXIDANT

One does not have to work long with Nile blue to become impressed by the observation that solutions of this dye in dilute buffers are of colloidal nature. It is well known that the chloride is much less soluble than the sulphate; but we were not prepared to find that concentrations of 0.0001 M of Nile blue sulphate cause appreciable deviations which tend to disappear only when the dye is diluted to the order of millionth molar or less.

In acid regions (pH 1 to 3) low concentrations of Nile blue gave solutions showing a definite Tyndall effect. We have not established

the threshold of dilution beyond which "true" solution is obtained; nor has it been possible to study other aspects as thoroughly as the problem deserves. The following discussion, however, will reveal some of the simpler factors bearing on the main theme.

The relation of pH to the concentration effect.—An aqueous solution of Nile blue sulphate (sample H) was made by warming 0.5 gm. of the dye in 750 c. c. of water. This was filtered twice through the same asbestos filter, the dye content determined by titration with titanous chloride, and the solution then diluted to 0.0015 molar concentration. This solution was then partly reduced (about 57 per cent) with hydrogen in the presence of platinized asbestos; then the fixed mixture of oxidant and reductant was carefully filtered through asbestos and stored in a nitrogen-protected buret.

The mixture was added in varying proportions as shown in Table 3 to citrate buffer No. 9, pH 4.881, ionic strength 0.23. The dilution of buffer was maintained constant by suitable additions of water. The potentials of the various mixtures established themselves almost at once to fairly constant values (within 0.5 mv.). It will be seen from Table 3 that with increasing concentrations of dye the equilibrium potentials become more negative. There is a 14-mv. shift with tenfold concentration from 1.4×10^{-5} to 1.4×10^{-4} M; and the change in potential is a linear function of the logarithm of the concentration. When the cells were opened for cleaning one to two hours after the commencement of the measurements, all the solutions showed a fine precipitate by the Tyndall effect. The most concentrated solution also contained moderate sized flocks of dark blue material.

TABLE 3.—*Nile blue sulphate—Effect of concentration*

[Sample H, 0.0015 M, about 57 per cent reduced; and mixture of oxidant+reductant added to 50 c. c. buffers Nos. 9 and 22, pH 4.881 and 8.642]

Mixture, c. c.	H_2O, c. c.	Mixture, final concentration	pH 4.881		pH 8.642
			E_h found	E_h calculated [1]	E_h found
0.5	4.5	0.000014 M	+0.0018	+0.0041	−0.1739
1.0	4.0	.000027 M	−.0023	−.0007	−.1759
2.0	3.0	.000055 M	−.0060	−.0058	−.1741
3.0	2.0	.000082 M	−.0086	−.0087	−.1725
5.0	0.0	.000136 M	−.0122	−.0123	−.1685

[1] Calculated from: Log concentration = $-4.61-60.1\ E_h$.

A similar experiment was performed in borate buffer No. 22, pH 8.642, ionic strength 0.10. The results are shown in the last column of Table 3. A marked negative drift in potential occurred in the most concentrated solution. In this case the first value (taken within 30 seconds with duplicate electrodes) is recorded. In the others, a peak negative value was attained within 2 to 3 minutes, the potentials then drifted slightly more positive for about 10 minutes, after which there followed a rapid drift to progressively more nega-

tive values. The first negative peak was the value selected in each of these cases. Excepting the most dilute solution and despite the lower precision in measurement the data show an almost linear relation between concentration and potential, with an 8-mv. shift for five-fold concentration. The interesting feature of this experiment is that the *direction* of potential change with concentration at pH 8.642 is opposite to that at pH 4.881. We shall refer to this point presently.

When the alkaline cells were opened for cleaning (3 hours after measurement), the solutions were pale blue in color and the more concentrated ones contained heavy flocks of precipitated dye. The most dilute contained a fine precipitate.

The excessively rapid drifts occurring in more alkaline solutions prevented an examination of the concentration effects in the region near pH 10 and 11, where all the oxidant would be present as the "free" base.

A further study of the concentration effect at pH 4.881 was made by diluting a Nile blue sulphate solution with deoxygenated buffer. In the electrode vessel was placed 50 c. c. of buffer, and after deaeration there were added 5 c. c. of a mixture of oxidant and reductant. The potential reached equilibrium at once and remained constant within 0.2 mv. for over 2 hours. The mixture in the vessel was then progressively diluted at constant pH and ionic strength (except for the small effects of the dye itself) by electrometric titration with a deoxygenated mixture of 50 c. c. buffer of pH 4.881 and 5 c. c. water. The concentration was thus varied from 13.6×10^{-5} to 7.5×10^{-5} M; the results are shown in Table 4 and Figure 1. When the electrode vessel was opened after the measurements, the dilute solution showed a definite Tyndall effect and a very fine precipitate was found after close examination.

TABLE 4.—*Dilution of Nile blue sulphate (sample H) with deoxygenated buffer No. 9, pH 4.881 diluted 50–5*

[Initial mixture: 50 c. c. buffer + 5 c. c. 0.0015 M dye, 37 per cent reduced]

Total volume c. c.	Total dye $[S_o] + [S_r]$, molar	E_h found	E_h calculated[1]
55	0.000136	−0.0123	−0.0123
57	.000132	−.0121	−.0122
59	.000127	−.0118	−.0119
60	.000125	−.0117	−.0118
62	.000121	−.0114	−.0115
65	.000116	−.0111	−.0112
68	.000110	−.0107	−.0108
71	.000106	−.0105	−.0106
76	.000099	−.0100	−.0101
80	.000094	−.0096	−.0097
86	.000087	−.0091	−.0091
95	.000079	−.0084	−.0084
100	.000075	−.0080	−.0080

[1] Log concentration = $-4.61 - 60.1\ E_h$

The logarithmic relationship between concentration and potential is followed very closely in this experiment and is described by the

familiar type equation: $y = ae^{-bx}$. On introducing numerical values for the constants the logarithmic form for the present case becomes:

$$\log \text{concentration} = -4.61 - 60.1\ E_h$$

It will be shown later that the effect is primarily dependent on the amount of oxidant present in solution. Strictly speaking, therefore,

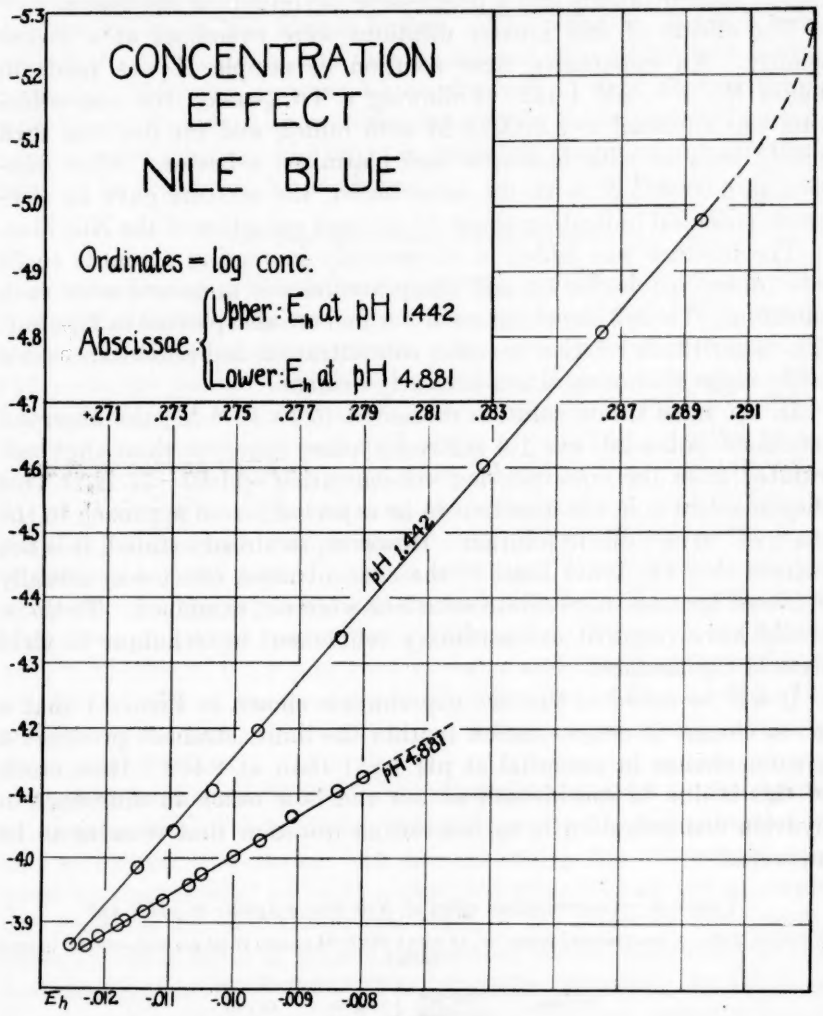


FIGURE 1

the total concentrations listed in the various tables should be corrected to *effective* concentrations; that is, to concentrations of oxidant alone. This has not been done in the instances cited in this paper, because the precise nature and mechanism of the relationship has not yet been satisfactorily established and because the general course

of the phenomenon is equally well brought out by the original, uncorrected data.

The last column of Table 4 shows the values of E_h calculated from the above equation. The agreement with the observed values is good. The application of this equation to the data for pH 4.881 in the preceding experiment, Table 3, shows good agreement for the higher concentrations and a progressive deviation for the lower.

The effects of still greater dilutions were examined at a higher acidity. An apparently clear solution of sample H was made in buffer No. 1 S, pH 1.442. Following a $TiCl_3$-assay, the concentration was adjusted to 0.000275 M with buffer, and the dye was then partly reduced with hydrogen and platinized asbestos. After dilution to 0.000025 M with the same buffer, the mixture gave an electrode potential indicating about 35 per cent reduction of the Nile blue.

The mixture was added in successively increasing amounts to 50 c. c. deaerated buffer 1 S and the potential was measured after each addition. Table 5 shows the results which are also plotted in Figure 1. The logarithmic relation between concentration and potentials over a wide range of concentration is clearly evident.

In the most dilute solution measured (5.4×10^{-6} M) the observed electrode potential was 1.6 millivolts more negative than that calculated from the equation, log concentration $= 11.52 - 57\ E_h$. This displacement is in the direction to be expected for an approach to the state of "true" dilute solution. However, as already stated, it is not certain that the lower limit of the concentration effect was actually attained because more dilute solutions were not examined. To do so would have required extraordinary refinement in technique to yield data of significance.

It will be noted in the two experiments shown in Figure 1 that a given change in concentration (within the limits studied) produces a greater change in potential at pH 4.881 than at 1.442. How much of this is due to specific salt action and how much to difference in hydrion concentration is an interesting question that remains to be answered.

TABLE 5.—*Concentration effect of Nile blue sulphate at pH 1.442*

[Titration of 50 c. c. deoxygenated buffer No. 1S with 0.000275 M sample H (35 per cent reduced) in same buffer]

Mixture, c. c.	Total dye $[S_r]+[S_o]$, molar	E_h observed	E_h calculated [1]
1	0.0000054	+0.2929	+0.2945
2	.0000106	.2895	.2894
3	.0000156	.2864	.2864
5	.0000250	.2827	.2828
10	.0000458	.2783	.2782
15	.0000635	.2757	.2757
20	.0000786	.2743	.2741
25	.0000905	.2731	.2730
30	.0001031	.2720	.2720

[1] Log concentration $= 11.52 - 57\ E_h$

The data, though incomplete, reveal the general outlines of what is probably the most important aspect of the concentration effect. In acid regions, increasing concentrations of dye produce a shift in electrode potential toward more negative values as if active oxidant were withdrawn from the solution; and in alkaline solution the reverse effect obtains. Consistent with the view that active oxidant is *ultimately* withdrawn is the repeated observation of a Tyndall effect in dilute solutions of Nile blue in buffers. (The reductant is much more soluble than the oxidant and may be left out of consideration for the present.) However, it must be emphasized at once that although withdrawal of oxidant by precipitation may be the important factor in concentrated solutions, the effect in moderate ranges of concentration can not be accounted for so simply.

SPECTROPHOTOMETRIC OBSERVATIONS

Another line of evidence bearing on this point is furnished by the spectrophotometric analysis of various concentrations of Nile blue in the visible region of the spectrum. An aqueous solution containing 665 parts per million (0.0018 M) of sample H gave through a layer 0.4 mm. thick the transmittance curve 1 shown in Figure 2. This curve is characterized by a sharp peak at 578 mμ and a definite hump near 630 mμ. The same solution when diluted to 16 p.p.m. (0.000044 M) gave through a layer 8.5 mm. thick the curve 2, which shows a peak at 595 mμ and another at 630 mμ. When diluted to 1 p.p.m. (0.0000027 M) and viewed through an 85 mm. layer, it gave curve 3, which shows a sharp peak at 633 mμ and an indefinite hump near 600 mμ. Two additional curves were obtained (not shown in the figure) with a dilution of 0.5 p.p.m., one in water and the other in buffer of pH 4.881. The curves were practically identical and each one had in addition to a main peak at 635 mμ, a definitely perceptible hump in the region of 600 mμ.

Modern studies on the relation of absorption spectra to the chemical constitution of dyes distinguish between rotational, vibrational, and electronic frequencies. In the molecules the vibrational frequencies are revealed as band absorption systems and not as single lines, because to each vibrational state of the molecule there corresponds a whole series of separate rotational states. According to the British Association Report, 1922, there is, generally speaking, a characteristic absorption band in the short infra-red region which is due to the molecule as a whole; furthermore, certain visible and ultraviolet bands are due to the conditions under which the molecules exists.

In Nile blue there is clearly evident the existence of at least two molecular phases or species, the proportions of which are conditioned by the amount of dye in solution. The phase characterized by the absorption band centered near 580 mμ is predominant in

concentrated solutions of Nile blue and that characterized by the absorption at 630 mμ is predominant in dilute solutions.

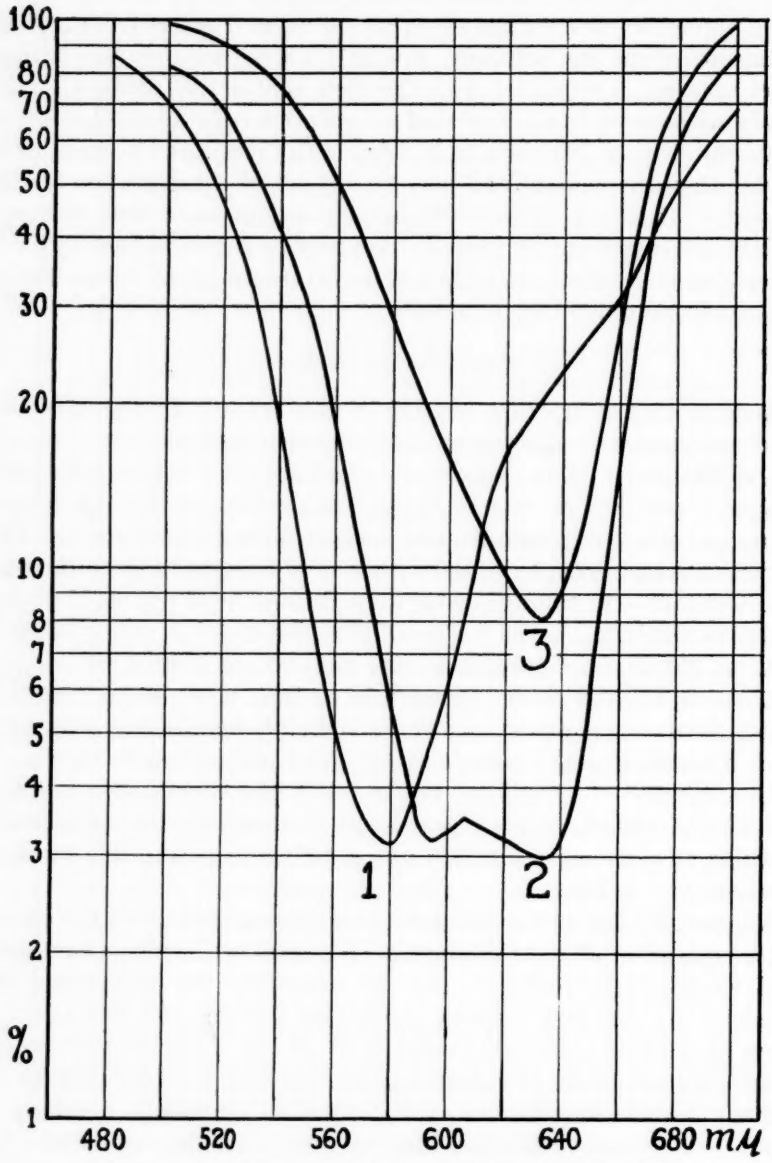


FIGURE 2.—Transmittance curves of aqueous solutions of Nile blue sulphate

Curve 1: Conc. 0.0018 M; solution thickness, 0.4 mm.
Curve 2: Conc. 0.000044 M; solution thickness, 8.5 mm.
Curve 3: Conc. 0.00000027 M; solution thickness, 85.0 mm.
(Ordinates = per cent transmittance; abscissae = wave length.)

These relations of concentration to color absorption are more or less well known. Formánek and Grandmougin (1909) mention this prop-

erty of Nile blue A and conclude that it is a hydrolytic dissociation of the dye by the water. Some of the general features of this phenomenon and the related problem of chromoisomerism are discussed by Dehn (1917) and by Holmes (1924). This controversial subject will not become clarified, however, until more decisive experiments can be devised to permit a choice between the various plausible hypotheses now current.

There is another aspect of the spectrophotometric analysis which seems to have escaped recognition up to now and which may have important consequences. We refer to the progressive shift toward the red end of the spectrum of the absorption band associated with concentrated solutions. This is to be seen in Figure 2 and is perhaps better illustrated in Table 6. The forty-fold dilution from 0.0018 M to 0.000045 M causes the peak of this characteristic band to move from 578 to 595 mμ. The shift is approximately a function of the logarithm of the total concentration. On the other hand, the predominant band for dilute solutions remains fixed at 630 mμ in this experiment for the ten-fold dilution from 0.00009 to 0.000009 M; but in the preceding experiment it was found that at still greater dilutions (2.7×10^{-6} to 1.4×10^{-6} M) this peak was centered at 633 to 635 mμ. This last small difference may be without significance, but the above shift of 17 mμ challenges thought.

TABLE 6.—*Nile blue sulphate (sample H). Spectrophotometric analysis in the visible spectrum showing effect of dilution*

Concentration, molar	Solution thickness	Transmittance and wave length at maximum absorption			
		T	mμ	T	mμ
	Millimeters	*Per cent*		*Per cent*	
0.0018	[1] 0.35	1.2	578	--------	[2]
0.0009	[1] 0.80	1.15	580	--------	[2]
0.00018	2.8	1.15	590	4.0	[1] 630
0.00009	5.4	1.25	592	2.4	630
0.000045	10.0	1.5	595	1.55	630
0.000009	40.0	--------	[2]	1.5	630

[1] Approximate.
[2] Hump without a definite peak.

Experiment shows that this shift is reversible. Three liters of the most dilute solution (0.000009 M) were concentrated to a volume of about 30 c. c. by boiling under reduced pressure in a stream of purified nitrogen. The concentrate was filtered to remove precipitated material, and the filtrate was examined spectrophotometrically. It was found that the single absorption band for "dilute" solution, centered at 630mμ, had changed to a double band with peaks at 630mμ and 597mμ.

It might be assumed that the observed shift is due merely to an overlapping dissymmetry of the stationary absorption band. This view is not entirely convincing, however, because each of the two bands has a high degree of symmetry in solutions where the other is almost suppressed.

The more obvious interpretation of the relative fixity of the absorption peak at 630 mμ and of the regular approach to it of the other peak is that the molecular species or tautomer predominant in concentrated solution changes its structure in stepwise fashion with dilution until finally it becomes identical with the presumably simple structure characteristic of dilute solutions. The only type of tautomer that can answer this description seems to be an associated molecule or a micelle whose complexity varies with the concentration of solute.

A final determination of this point must be left to further experimentation. If the above interpretation is correct then molecular weight estimations of Nile blue by the diffusion method should show increasing molecular weight with increasing concentration.

The evidence thus far obtained on the concentration effect may be summed up as follows:

(1) Nile blue in concentrations of the order of millionth molar in water approaches obedience to the laws of dilute solutions.

(2) In moderate concentrations, Nile blue acts as if it associated to form micellar structures. The range of "moderate" concentrations is indefinite, but it certainly extends from 1×10^{-5} to 5×10^{-4} molar.

(3) This micelle formation occurs in acid solutions down to about pH 7. In more alkaline regions the process of aggregation seems to be reversed. The evidence therefore suggests that this tautomerization is related to the concentration of *the ionized oxidant* (Ox^+). Micelle formation is also reversed, of course, with dilution.

(4) In concentrated solution, the association of Nile blue molecules proceeds apparently to the stage of precipitation. The latter would naturally be enhanced by the presence of salts.[5]

OXIDATION-REDUCTION POTENTIALS IN "DILUTE" SOLUTION

Electrometric titrations of the chloride.—A stock 0.000275 M solution of the oxidant was made in water, heat (60°) being used to aid solution. The dye was then reduced to a clear yellowish color with hydrogen and platinized asbestos, and stored in a nitrogen-protected buret. Of this, 5 c. c. were added to 50 c. c. deoxygenated buffer and titrated with benzoquinone dissolved in equally diluted buffer. Table 7 shows a titration made at pH 1.394, which gave a sharp endpoint at

[5] In the titanium assay of Nile blue it is necessary to wait several minutes between successive additions of reducing agent near the end in order to obtain reproducible endpoints. We have occasionally observed in the titration of colloidal dyes, Meldola's blue in particular, that the electrometric endpoint was as low as 50 per cent of the expected.

40.4 c. c. With this as a basis, the E_o' values calculated from the observed potentials are those of the 5th column of Table 7. The distribution of values suggests that the titration had been begun on a solution already partially oxidized. By assuming about 5.4 per cent initial oxidation, i. e., the equivalent of 2.3 c. c. quinone already present, there are obtained the values shown in the last column of Table 7 (average $E_o' = 0.2808$).

Attention should be called to a deficiency in our treatment of the data. Strictly speaking, proper corrections should have been introduced for acid formed during the titration and for the variation in concentration of the oxidant. The deviations due to acid formation are very small and in the direction opposite to the general trend of values in this and subsequent tables. Deviations due to concentration change of the oxidant increase from zero to a variable maximum (depending on pH) of a few millivolts and are in the same direction as those observed. However, calculations indicate that effects of concentration change in these experiments are secondary to the more pronounced effect of the apparently unavoidable initial oxidation of the reductant. (Compare Tables 7 and 8 with Table 11.) It is for this reason that refinements were ignored.

TABLE 7.—*Titration of reduced Nile blue chloride with benzoquinone in sulphate buffer No. 1S, pH 1.394*

[Initial dye concentration 0.000025 M]

Quinone, c. c.	Oxidation, per cent	$0.03006 \times \log \frac{[S_r]}{[S_o]}$	E_h	E'_o	Quinone, corrected c. c.	E'_o corrected
2	4.95	+0.0386	+0.2493	+0.2879	4.3	(+0.2779)
4	9.90	.0288	.2566	.2854	6.3	(.2795)
5	12.38	.0255	.2593	.2848	7.3	.2799
6	14.85	.0228	.2616	.2844	8.3	.2802
7	17.33	.0204	.2637	.2841	9.3	.2804
8	19.80	.0183	.2656	.2839	10.3	.2806
9	22.28	.0163	.2673	.2836	11.3	.2806
10	24.75	.0145	.2690	.2835	12.3	.2808
11	27.23	.0128	.2704	.2832	13.3	.2808
12	29.70	.0112	.2719	.2831	14.3	.2809
13	32.18	.0097	.2733	.2830	15.3	.2809
15	37.13	.0069	.2759	.2828	17.3	.2809
18	44.55	.0029	.2796	.2825	20.3	.2809
20	49.50	+.0003	.2821	.2824	22.3	.2809
22	54.46	−.0023	.2845	.2822	24.3	.2809
25	61.88	−.0063	.2883	.2820	27.3	.2808
27	66.83	−.0091	.2910	.2819	29.3	.2808
30	74.26	−.0138	.2956	.2818	32.3	.2808
31	76.73	−.0156	.2973	.2817	33.3	.2808
32	79.21	−.0175	.2992	.2817	34.3	.2808
33	81.68	−.0195	.3012	.2817	35.3	.2808
34	84.16	−.0218	.3036	.2818	36.3	.2810
35	86.63	−.0244	.3062	.2818	37.3	.2810
36	89.11	−.0274	.3093	.2819	38.3	.2810
37	91.58	−.0312	.3130	.2818	39.3	.2811
38	94.06	−.0361	.3181	.2820	40.3	(.2813)
39	96.53	−.0434	.3251	.2817	41.3	(.2809)
40.4	100.00				42.7	

Average = 0.2808

TABLE 8.—*Titration of reduced Nile blue chloride with benzoquinone in acetate buffer No. 2A, pH 5.191*

[Initial dye concentration 0.000025 M]

Quinone, c. c.	Oxidation, per cent	$0.03006 \times \log \frac{[S_r]}{[S_o]}$	E_h	E'_o	Quinone, corrected c. c.	E'_o corrected
2	9.76	+0.0290	−0.0472	−0.0182	2.8	−0.0225
4	19.51	.0185	−.0383	−.0198	4.8	−.0222
6	29.27	.0115	−.0319	−.0204	6.8	−.0220
7	34.15	.0086	−.0292	−.0206	7.8	−.0220
8	39.02	.0059	−.0267	−.0209	8.8	−.0221
9	43.90	+.0032	−.0242	−.0210	9.8	−.0221
10.5	51.22	−.0006	−.0207	−.0213	11.3	−.0223
12	58.54	−.0045	−.0171	−.0216	12.8	−.0224
13	63.41	−.0072	−.0145	−.0217	13.8	−.0225
14	68.29	−.0100	−.0118	−.0218	14.8	−.0225
15	73.17	−.0131	−.0088	−.0219	15.8	−.0226
17	82.93	−.0206	−.0013	−.0219	17.8	−.0225
19	92.68	−.0331	+.0112	−.0219	19.8	−.0225
20.5	100.00				21.3	

Average = −0.0223

Titration of another stock solution was made in acetate buffer of pH 5.191, yielding an average $E'_o = -0.0223$ (Table 8). Here again was found evidence of an initial oxidation to the extent of about 2.75 per cent. In both cases the stock reductant solution showed no visible evidence of recoloration. Considering the rather high dilution of the dye, small contaminations by oxygen which would ordinarily be insignificant were undoubtedly responsible for the effects observed. This point deserves emphasis.

A repeat titration was made at pH 1.394 using quinone of double strength. The purpose was to obtain a better orientation value for the final study of the variation of E'_o with pH. This time a small amount of oxygen was allowed to enter the reservoir and the contents were thoroughly stirred with deoxygenated nitrogen until homogeneous. The reductant was clear yellow with a faint greenish tint that was visible only through a thick layer. The titration results are shown in Table 9, giving an average $E'_o = 0.2795$. The amount of initial oxidation in this case was calculated to be 7.4 per cent.

The E'_o values obtained in the last two titrations were used to fix the position of the E'_o : pH curve.

TABLE 9.—*Titration of reduced Nile blue chloride with benzoquinone in sulphate buffer No. 1S, pH 1.394*

[Initial dye concentration 0.000025 M]

Quinone, c. c.	Oxidation, per cent	$0.03006 \times \log \frac{[S_r]}{[S_o]}$	E_h	E'_o	Quinone, corrected c. c.	E'_o corrected
2	8.89	+0.0304	+0.2567	+0.2871	3.8	+0.2787
4	17.78	.0200	.2645	.2845	5.8	.2796
6	26.67	.0132	.2700	.2832	7.8	.2798
7	31.11	.0104	.2725	.2829	8.8	.2799
8	35.56	.0078	.2747	.2825	9.8	.2798
9	40.00	.0053	.2768	.2821	10.8	.2797
10	44.44	.0029	.2789	.2818	11.8	.2797
11	48.89	+.0006	.2810	.2816	12.8	.2796
12	53.33	−.0017	.2830	.2813	13.8	.2794
13	57.78	−.0041	.2852	.2811	14.8	.2794
14	62.22	−.0065	.2874	.2809	15.8	.2793
16	71.11	−.0118	.2924	.2806	17.8	.2792
18	80.00	−.0181	.2985	.2804	19.8	.2792
20	88.89	−.0271	.3075	.2804	21.8	.2792
21	93.33	−.0344	.3150	.2806	22.8	.2795
22.5	100.00				24.3	

Average=0.2795

RELATION OF E'_o TO pH FOR NILE BLUE CHLORIDE

A fixed mixture was made of oxidant and reductant (total concentration, 0.000275 M). This was found to be 65.2 per cent oxidized by measurement of 5 c. c. of the mixture in 50 c. c. of buffers pH 1.394 and 5.191. The fixed mixture was then added in the same proportion to a large series of buffers and the resulting potentials observed. The results are shown in Table 10. Unfortunately, most of the buffers contained chloride ions which exerted a pronounced salting-out effect on this poorly soluble colloidal dye. It was therefore necessary in most cases to accept the first peak negative potentials obtained. This was the case in buffers Nos. 8, 10, 13, 14, 15, 16, 22, 24, 24½, 26, and 28. Drifts of potentials were so rapid that initial values or those obtained within the first two minutes had to be chosen in buffers 7 and 9. Observations in buffers 5 and 6 were rejected because drifts were excessive and erratic from the very start. Stable plateau values were obtainable in the remaining cases. Of particular note is the observation that apparent true equilibrium values were obtained in the two acetate buffers used, the potentials remaining constant to within 0.1 mv. for at least one hour.

TABLE 10.—*Nile blue chloride.—Relation of E'_0 to pH (Concentration 0.000025 molar)*

Buffer No.	pH	E'_0 observed	E'_0 calculated[1]	Deviation
1S	1.394	+0.279	+0.280	−0.001
2S	1.722	.255	.250	+.005
3S	2.375	.186	.192	−.006
1	1.076	.313	.309	+.004
7	3.860	.068	.066	+.002
8	4.413	.027	.027	.000
1A	4.598	+.015	+.014	+.001
2A	5.191	−.022	−.023	+.001
9	4.898	−.002	−.005	+.003
10	5.317	−.036	−.031	−.005
13	5.876	−.064	−.064	.000
14	6.256	−.090	−.085	−.005
15	6.737	−.111	−.110	−.001
16	7.424	−.144	−.139	−.005
20	8.065	−.161	−.161	.000
22	8.646	−.180	−.180	.000
23	9.278	−.198	−.203	+.005
23½	9.596	−.210	−.215	+.005
24	10.26	−.252	−.248	−.004
24½	11.03	−.291	−.291	.000
26	11.51	−.316	−.320	+.004
28	12.29	−.360	−.367	+.007

[1] Calculated by means of equation (11) using

$E'_{pH\ 0} = +0.406$. $K_{r1} = 1.20 \times 10^{-4}$. $pK_{r2} = 6.90$.
$K_{ob} = 9.94 \times 10^{-4}$. $K_w = 1.88 \times 10^{-14}$. $pK_{r3} = 3.92$.
$K_{r2} = 1.26 \times 10^{-7}$. $pK_{ob} = 4.025$. $pK_w = 13.725$.

The calculated values for E'_0 are given in Table 10 and are shown in Figure 3 as black circles. The calculations are based on the assumption that the effect of dye concentration (in this instance 0.000025 molar) is negligible. It should be emphasized that this is an arbitrary assumption employed merely for the preliminary outline of the Nile blue system. The experimental evidence shows fairly definitely that the concentration effect is still appreciable at concentrations of 0.000005 molar Nile blue in the buffers used. Just how far the dilution may be pushed and still yield significant potentials by the potentiometric method remains to be determined; but it is clear that the experimental difficulties will increase considerably as the primary active system is diluted to a point where its concentration approaches in order of magnitude that of active contaminants from the solvent, container, electrodes, and minute oxygen leaks.

ELECTRODE MEASUREMENTS OF NILE BLUE SULPHATE

The more soluble sulphate of Nile blue was used in the following experiments. The dye (sample H) was dissolved in *cold* water, assayed with $TiCl_3$ and then diluted to make a 0.000275 M stock solution. The clear yellow reduced solution when titrated with benzoquinone at pH 1.442 gave results indicating the presence of 5 to 7 per cent oxidant in the reductant.

TABLE 11.—*Titration of Nile blue sulphate (sample H) with reduced anthraquinone β-sulphonate in sulphate buffer No. 1S, pH 1.442*

[Initial dye concentration 0.000025 M]

Reduced anthraquinone, c. c.	Reduction, per cent	$0.03006 \times \log \frac{[S_r]}{[S_o]}$	E_h	E'_o	Deviation from +0.2746
2	5.85	+0.0363	+0.3119	+0.2756	+0.0010
4	11.70	−.0264	.3007	.2743	−.0003
6	17.54	−.0202	.2942	.2740	−.0006
8	23.39	−.0155	.2895	.2740	−.0006
10	29.24	−.0115	.2857	.2742	−.0004
12	35.09	−.0080	.2824	.2744	−.0002
14	40.94	−.0048	.2793	.2745	−.0001
16	46.78	−.0017	.2763	.2746	.0
18	52.63	+.0014	.2733	.2747	+.0001
20	58.48	.0045	.2701	.2746	.0
22	64.33	.0077	.2670	.2747	+.0001
24	70.18	.0112	.2634	.2746	.0
26	76.02	.0151	.2594	.2745	−.0001
28	81.87	.0197	.2548	.2745	−.0001
30	87.72	.0257	.2487	.2744	−.0002
32	93.57	.0350	.2395	.2745	−.0001
34.2	100.00				

It was decided, therefore, to obtain a titration of the oxidant. The titrating agent was anthrahydroquinone-β-sulphonate obtained by the reduction of the sodium salt of anthraquinone–β–sulphonate ("silver salt") with hydrogen in the presence of platinized asbestos. The titration proceeded smoothly, giving prompt equilibrium potentials and a fairly good endpoint. The anthraquinone-β-sulphonate system overlaps the Nile blue system only slightly at pH 1.442 and estimation of the endpoint was not difficult. Table 11 shows the results of this titration; the fair agreement with the theoretical curve is indicated by the small deviations of the E'_o values from 0.2746 volt.

RELATION OF E'_o TO pH FOR NILE BLUE SULPHATE

The procedure was the same as that for the chloride. The fixed mixture was added in 5 c. c. portions to 50 c. c. of each of the various buffers. The potentials obtained were of the same order of stability as those of the Nile blue chloride system. The results are given in Table 12 and are shown in Figure 3 as circles. It will be seen that the chloride series and the sulphate series are in general agreement. The deviations from the calculated values are rather large in several instances, but the essential relations are quite clear and sufficient for this exploratory study.

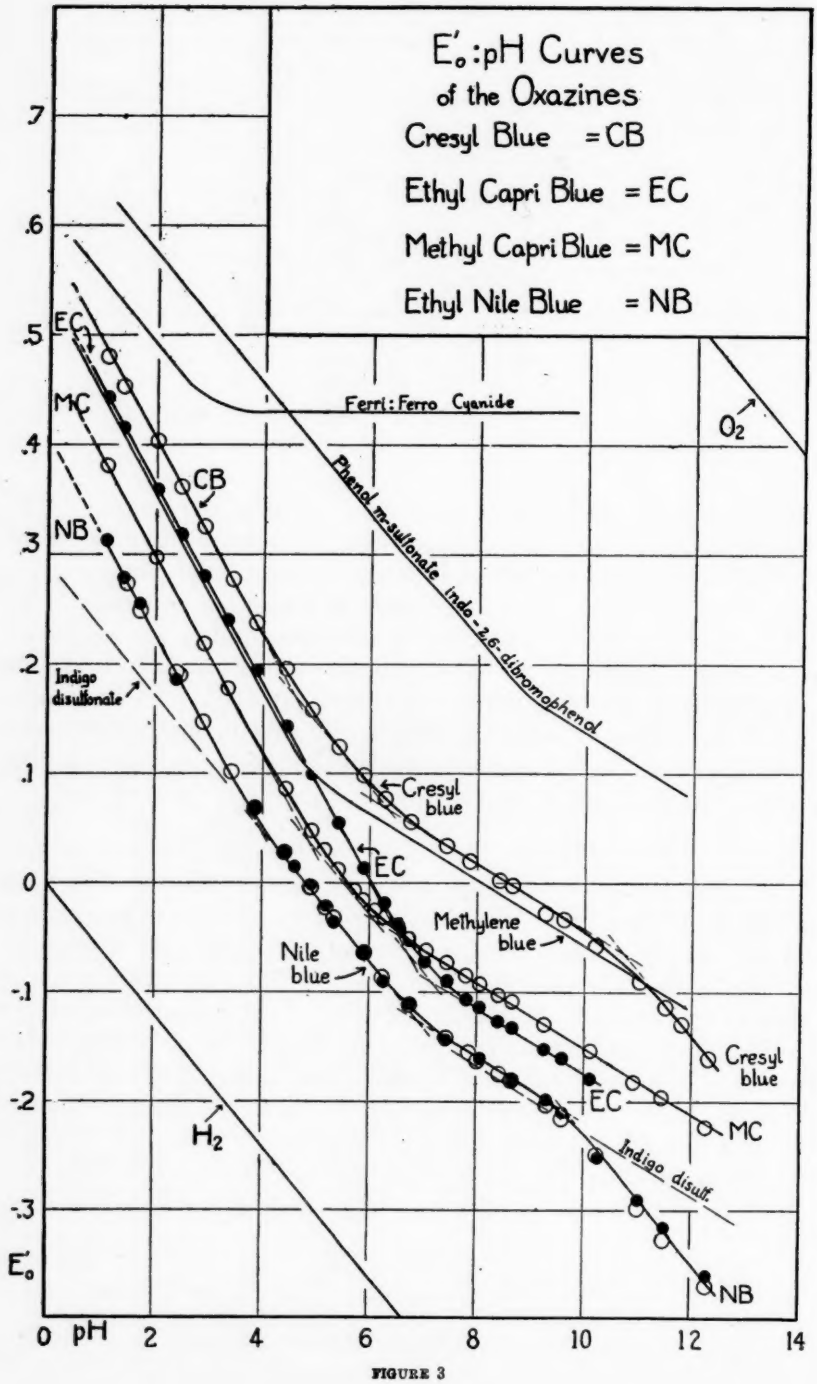

E'o:pH Curves
of the Oxazines
Cresyl Blue = CB
Ethyl Capri Blue = EC
Methyl Capri Blue = MC
Ethyl Nile Blue = NB
Ferri:Ferro Cyanide
O2
Phenol m-sulfonate indo-2,6-dibromophenol
Indigo disulfonate
Cresyl blue
Methylene blue
Nile blue
Indigo disulf.
H2
E'o
pH
EC
MC
CB
NB
.7
.6
.5
.4
.3
.2
.1
0
-.1
-.2
-.3
0
2
4
6
8
10
12
14

FIGURE 3

TABLE 12.—*Nile blue sulphate (sample H)—Relation of E'_o to pH (Concentration 0.000025 M)*

Buffer No.	pH	E'_o observed	E'_o calculated[1]	Deviation
1S	1.442	+0.275	+0.275	0.000
2S	1.722	.249	.250	−.001
3S	2.375	.193	.191	+.002
4	2.451	.190	.185	+.005
5	2.879	.147	.147	.000
6	3.421	.102	.100	+.002
7	3.860	.069	.065	+.004
8	4.413	+.028	+.026	+.002
9	4.898	−.004	−.006	+.002
2A	5.191	−.023	−.024	+.001
10	5.317	−.032	−.032	.000
13	5.876	−.065	−.065	.000
14	6.256	−.088	−.086	−.002
15	6.737	−.113	−.111	−.002
16	7.424	−.141	−.140	−.001
17	7.849	−.156	−.155	−.001
20	8.065	−.163	−.162	−.001
21	8.411	−.175	−.173	−.002
22	8.646	−.181	−.181	.000
23	9.278	−.203	−.204	+.001
23½	9.596	−.216	−.216	.000
24	10.24	−.249	−.248	−.001
24½	11.03	−.298	−.292	−.006
26	11.51	−.327	−.321	−.006
28	12.29	−.370	−.268	−.002

[1] Calculated by means of equation (11) using the constants of Table 10.

OXIDATION-REDUCTION POTENTIALS IN "CONCENTRATED" SOLUTION

The measurements to be reported in this section were made upon solutions of Nile blue sulphate having a final total concentration of 0.000136 molar. The purpose was to determine the general direction and magnitude of displacement of the oxidation-reduction potentials from those found in the preceding section for "dilute" solution (0.000025 M). A more thorough examination of this question is considered beyond the scope of the present investigation.

Nile blue sulphate (sample H), 0.5 gm. was ground up in 750 c. c. water, filtered, assayed with $TiCl_3$, and then diluted to give a 0.0015 M stock solution. A portion of the latter was reduced to a clear yellow with hydrogen in the presence of platinized asbestos. Five c. c. of the reductant were added to 50 c. c. buffer No. 9, pH 4.881 and titrated with benzoquinone dissolved in equally diluted buffer No. 9. The initial dye concentration was 0.000136 M, the final 0.000097 M. The titration proceeded smoothly, and the potentials after each addition of quinone attained constancy within two minutes. The results are shown in Table 13. When the titration was completed the solution was clear blue, but a definite Tyndall effect was evident when observed under a 250-watt Mazda lamp. However, the titration curve was smooth and did not show a break or marked displacement at the end such as would be expected if appreciable precipitation of oxidant had occurred. There was present at the *beginning* of the titration a displacement such as was found in dilute solutions and interpreted as due to initial oxidation of a portion of the reductant.

When correction was made for 6.3 per cent initial oxidation the data lined up as shown in the last column of Table 13 to a fairly constant value of −0.0136 volt for E'_0. This value will be used in fixing the position of the E'_0:pH series.

TABLE 13.—*Titration of reduced Nile blue sulphate (sample H) with benzoquinone in buffer No. 9, pH 4.881*

[Initial dye concentration, 0.000136 M]

Quinone c. c.	E_h	E'_0	Quinone corrected c.c.	E'_0 corrected
2	−0.0382	−0.0080	3.5	−0.0153
3	−.0331	−.0088	4.5	−.0141
4	−.0292	−.0093	5.5	−.0135
6	−.0235	−.0105	7.5	−.0134
8	−.0185	−.0109	9.5	−.0132
10	−.0141	−.0114	11.5	−.0132
12	−.0097	−.0117	13.5	−.0132
14	−.0052	−.0120	15.5	−.0134
16	−.0002	−.0124	17.5	−.0136
18	+.0061	−.0126	19.5	−.0136
20	+.0157	−.0125	21.5	−.0135
21	+.0239	−.0124	22.5	−.0133
22.3			23.8	

Average = −0.0136

Measurements of mixtures of oxidant and reductant at pH 4.881 gave the data of Table 14. In the last two columns of this table are given the corrections for 6.3 per cent oxidant assumed to correct for the quinone titration of the same solution. The agreement with the titration is fair.

TABLE 14.—*Nile blue sulphate (sample H.) Mixtures of oxidant and reductant at pH 4.881*

[Total oxidant and reductant 0.000136 molar]

Ratio $\frac{[S_r]}{[S_o]}$	E_h	E'_0	Ratio $\frac{[S_r]}{[S_o]}$ corrected	E'_0 corrected
$\frac{40}{60}$	−0.0080	−0.0133	$\frac{37.48}{62.52}$	−0.0142
$\frac{50}{50}$	−.0126	−.0126	$\frac{46.85}{53.15}$	−.0142
$\frac{60}{40}$	−.0169	−.0116	$\frac{56.00}{43.78}$	−.0136

Average = −0.0140

A number of other titrations in "concentrated" solution were made but they will not be cited. All of them gave fairly acceptable titration curves and practically stoichiometrical endpoints. If our conclusion is true that Nile blue in "concentrated" solution aggregates to complex micelles, then the fact that there is obtained prompt equilibrium and stoichiometrical equivalence at all points on the titration curve *makes it appear that the oxidation-reduction reactivity of the components*

in the complex molecule is preserved intact. The difference in energy of reduction between concentrated and dilute solutions of the dye must represent the energy of aggregation.

The variation of E_h with pH was determined with a fixed mixture of 0.000136 molar total concentration, 37 per cent reduced. The difficulties in obtaining the measurements were considerable, since in many cases rapid precipitation occurred. However, by working quickly and repeating measurements at important points, a fair degree of order was finally obtained. Nevertheless, the values given must be considered with caution, since in many cases the measurements obtained were by no means experimental equilibrium values in the accepted sense.

At pH 2.448 there occurred a heavy precipitate; considerable precipitation also occurred in the phosphate and borate buffers; and in alkaline regions the red base flocked out so extensively as to leave the solutions almost colorless.

In general, the potentials established themselves quickly in acid solutions to values that remained constant within 1 millivolt for almost an hour. Where there was rapid precipitation, we chose the initial or the peak negative values as the more probable. The results are given in Table 14 A.

TABLE 14 A.—*Nile blue in "concentrated solution"—Relation of E'_o to pH*

[Total concentration of oxidant and reductant 0.000136 M]

Buffer No.	pH	E'_o observed
1	1.397	+0.269
2	1.707	.241
4	2.448	.177
6	3.351	.098
7	3.883	.055
8	4.399	+.018
9	4.881	−.014
10	5.491	−.051
11	5.907	−.076
14	6.444	−.102
15	6.828	−.119
16	7.393	−.139
17	7.875	−.153
20	8.083	−.155
21	8.404	−.165
22½	8.966	−.178
23	9.282	−.191
24	10.23	−.246
24½	11.02	−.294
26	11.52	−.328
28	12.39	−.371

Serious obstacles to a proper interpretation of the peculiarities of the E'_o: pH curve for "concentrated" solutions of Nile blue are imposed by the great uncertainties and irregularities of the measurements in alkaline regions from pH 7 on. For the present, therefore, the experimental data of Table 14 A are recorded for purposes of reference without further analysis. The general direction and magni-

tude of the displacement due to micelle formation by the polar oxidant is evident from a comparison of the data in Tables 14 and 14 A.

Additional comment will be found in the general discussion.

ELECTRODE EQUATIONS

The characteristics of the E'_0: pH curve and the chemical structure furnish the means for a rational formulation of the experimental data. Structurally the oxazines are analogous to the thiazines, and there is good evidence to indicate that the alkyl amino group has a distinct polar valence comparable to that of NH^+_4. On reduction, this valence is neutralized by an electron and there is also created a virtual anion at the bridging nitrogen.

The oxidation-reduction process may, therefore, be expressed as follows:

$$Ox^+ + 2\epsilon \rightleftarrows Red^-$$

and the corresponding electrode equation [6] is (at 30° C.)

$$E_h = C - 0.03006 \log \frac{[Red^-]}{[Ox^+]} \quad (1)$$

Nile blue in dilute solution.—In the formulation of the data for Nile blue at 0.000025 M the simplifying assumption will be made that concentration effects are negligible. This is permissible in the present instance because uncertainties of electrode potentials, especially in the alkaline and neutral regions, were often greater than the possible displacement due to the concentration effect.

The total concentration of reductant in its various forms is

$$[S_r] = [Red^-] + [H\ Red] + [H_2\ Red^+] + [H_3\ Red^{++}] \quad (2)$$

and the various forms are related to their dissociation constants according to the following equilibrium equations:

$$\frac{[Red^-][H^+]}{[H\ Red]} = K_{r1} \text{ (equivalent to binding } H^+ \text{ at bridging nitrogen)} \quad (3)$$

$$\frac{[H\ Red][H^+]}{[H_2\ Red^+]} = K_{r2} \text{ (addition of } H^+ \text{ to amino nitrogen)} \quad (4)$$

$$\frac{[H_2\ Red^+][H^+]}{[H_3\ Red^{++}]} = K_{r3} \text{ (addition of } H^+ \text{ to diethylamino nitrogen)} \quad (5)$$

By substituting (3), (4), and (5), in (2) there is obtained

$$[Red^-] = \frac{[S_r]K_{r1}K_{r2}K_{r3}}{K_{r1}K_{r2}K_{r3} + K_{r2}K_{r3}[H^+] + K_{r3}[H^+]^2 + [H^+]^3} \quad (6)$$

[6] See first and second papers of this series.

Similarly, the total concentration of the oxidant may be written:

$$[S_o]=[Ox^+]+[OxOH] \quad (7)$$

where $\frac{[Ox^+][OH^-]}{[OxOH]}=K_{ob}$ (dissociation of polar group of oxidant) (8*a*)

Introducing the ionic product of water (8*a*) becomes

$$\frac{[Ox^+]K_w}{[OxOH][H^+]}=K_{ob} \quad (8)$$

Substituting (8) in (7) there is obtained

$$[Ox^+]=\frac{[S_o][H^+]K_{ob}}{[H^+]K_{ob}+K_w} \quad (9)$$

The electrode equation (1) then becomes

$$E_h=C-0.03006 \log \left[\frac{[S_r]}{[S_o]}\times\frac{K_{r1}K_{r2}K_{r3}}{K_{ob}} \times\frac{[H^+]K_{ob}+K_w}{K_{r1}K_{r2}K_{r3}[H^+]+K_{r2}K_{r3}[H^+]^2+K_{r3}[H^+]^3+[H^+]^4}\right] \quad (10)$$

Let it be assumed, as indicated in equation (3), that the bridging nitrogen in the reductant fixes H^+. Consequently K_{r1} is negligibly small and the product $K_{r1}K_{r2}K_{r3}[H^+]$ is negligible in the sum. Then assembling constants under E_o and simplifying as indicated, *the electrode equation for Nile blue in dilute solution* becomes

$$E_h=E_o-0.03006 \log \frac{[S_r]}{[S_o]}-0.03006 \log \frac{[H^+]K_{ob}+K_w}{K_{r2}K_{r3}[H^+]^2+K_{r3}[H^+]^3+[H^+]^4} \quad (11)$$

The values of K_{r2} and K_{r3} are known to be of low order magnitude (<1); therefore when $[H^+]=1$ and $[S_r]=[S_o]$,

$$E'_{pH\,0}=E_o-0.03006 \log K_{ob}$$

and since $E'_{pH\,0}$ is determinable by graphic extrapolation of the 0.09-slope in the acid region, E_o can be evaluated. Equation (11) can then be employed for computing the E'_o:pH curve, which is represented by the solid line NB running through the experimental points in Figure 3.

DISSOCIATIONS

Inspection of the E'_o:pH curve of Nile blue shows three distinct bends representing detectable dissociations at pH 3.92, 6.9, and 9.7. The direction of the bends at pH 3.92 and 6.9 denotes dissociations in the reductant, and that at pH 9.7 denotes dissociation of the relatively strong polar diethylamino group (*cf.* Paper XIV). The simple amino group in Nile blue is such a weak base that its dissociation is

not detectable in the range of pH studied. These dissociations may be determined independently by titration, but they have been evaluated for present purposes by graphical analysis of the E'_o:pH curve.

The dissociation of the polar group in the oxidant occurs at pH 9.7, hence

$$pK_{ob} = pK_w - pH = 13.725 - 9.7 = 4.025$$

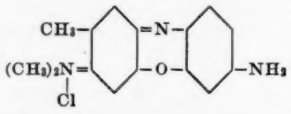
This is, of course, a calculation value which happens to fit the data. The precision of the measurements actually leaves the figure in the second decimal place in doubt.

In the reductant, the dissociation occurring at pH 3.92 is assignable to the simple amino group, $pK_{r2} = 3.92$, and that at pH 6.9 to the diethylamino group, $pK_{r3} = 6.90$.

In the case of Nile blue sulphate, it may be mentioned incidentally, if our interpretation of the chemical analyses is correct, this salt is an acid salt.

Brilliant Cresyl Blue

This compound as first prepared by Bender (English patent No. 1390, 1892), was the dimethylamino derivative and was formed by



Dimethyldiaminotoluphenoxazine chloride

the condensation of mononitroso-*m*-dimethylamino-*p*-cresol with *m*-phenylene diamine. The patent specifications also mention the diethyl homologue which is the compound described in Rowe's *Colour Index* as No. 877, brilliant blue C, cresyl blue BBS, 2 RN, and brilliant cresyl blue BB.

In the current biological literature, the compound (when its structure is specified) is described as the *diethyl* homologue; but there is reason to suspect that this is not usually the case. The few commercial products that we have examined possess the properties of the *dimethyl* homologue. A communication from the National Aniline and Chemical Co.[7] states that their brilliant cresyl blue is the dimethylamino compound. Our studies have been on the latter compound; and it should therefore be clear at the outset that this report deals with the properties of the dimethyl and not the diethyl homologue. It will be referred to hereinafter as *cresyl blue*.

SYNTHESIS, PURIFICATION, ANALYSIS, AND PROPERTIES

Owing to the difficulties in preparing sufficient amounts of the intermediate and in the proper synthesis of this dye, the present studies

[7] We express herewith our appreciation for the friendly cooperation of Dr. J. D. Kerr, of the National Aniline and Chemical Co., in submitting this information.

were made mainly on commercial products, purified and unpurified. Small quantities were synthesized to confirm the identity of the commercial samples.

The dye is prepared by condensing the hydrochloride of nitroso-dimethylamino-*p*-cresol with *p*-phenylene diamine in alcoholic solution from which cresyl blue crystallizes on cooling. With proper precautions against the presence of excess moisture in the mix, the resulting product yields a fair analysis and serves satisfactorily as a stain; but electrode measurements indicate the presence of an appreciable amount of an oxidizing impurity.

Purification of the cresyl blue by recrystallization from water has proved rather costly in material and of dubious value. The reason for this will be made apparent presently. A preparation which yielded fairly good electrometric data was obtained by the following procedure.

A 10-gram lot (No. 4482, National Aniline and Chemical Co.) was triturated with a small amount of cold water, dissolved in a minimum of warm (50°) water, and filtered. The solution was then almost saturated with sodium chloride and chilled to 0° or below, whereupon the dye separated out. The product was filtered off and dried in a vacuum desiccator. Each step was carried out as rapidly as possible to limit the contact of the dye with water. The resulting product (sample R) contained, of course, NaCl as impurity, but it proved more satisfactory electrometrically than the original. The analysis is shown in Table 15. The ash obtained, 39.30 per cent, is assumed to be admixed NaCl; the 24.82 per cent excess chlorine corresponds to 40.92 per cent NaCl, and the 1.62 per cent difference is possibly due to loss during incineration. On the basis of the nitrogen content, sample R is 55.00 per cent pure. This leaves a difference of 4.08 per cent unaccounted for.

Sample A, for which we are indebted to Dr. W. C. Holmes, of the Color Laboratory, United States Department of Agriculture, was a specimen recrystallized from water. It yielded a fair analysis for nitrogen, but the chlorine content was somewhat too high. The moisture content was also somewhat above that calculated (11.34 per cent) for two moles of water of crystallization. This preparation was the basis for many of our preliminary measurements. It was found to contain small amounts of active impurities which, however, did not seriously affect the general results of the electrode measurements.

Sample C (vital stain, lot No. 3955, National Aniline and Chemical Co.) is a representative of several commercial products not repurified by us. The analysis for nitrogen was close to the theoretical, but the chlorine content was much too high. No ash was obtainable from this specimen; hence the excess chlorine could not have been due to NaCl.

28131—31——5

TABLE 15.—*Analyses of cresyl blue chloride, $C_{15}H_{16}N_3OCl$; mol. wt., 289.61*

Sample	H_2O	Nitrogen	Chlorine	Ash
A	12.34	14.26	12.48	
C	8.70	14.49	14.42	0.0
R	5.21	7.98	31.55	39.30
Calculated (dry basis)		14.51	12.24	0.0

Cresyl blue in aqueous buffered solutions has a blue color from pH 1 down to pH 10. In 0.2 M HCl the color is lighter blue; in normal HCl the color is lavender and in 2 M HCl reddish; in 0.2 M NaOH the color is red which finally fades to a dull gray.

The dye is reduced by $Na_2S_2O_4$ from blue to colorless in fresh solutions of the pure compound. When the dye has been in contact with water for 10 to 15 minutes, the reduction proceeds from the blue to a red stage and then to a colorless one. Reduced solutions of cresyl blue at pH 7.4 to 10 are easily reoxidized by shaking with air; but in acid solutions down to pH 6.4 shaking with air did not result in the reappearance of the blue color of the oxidant for at least 30 minutes. Ferricyanide oxidized the reductant apparently instantly and completely at all points on the pH scale.

Solutions of the order of 0.001 molar cresyl blue in water are a clear blue when freshly prepared, but they develop a scum with a green metallic luster on standing and acquire with time a progressively redder tinge. Boiling cresyl blue in water for several hours under a reflux condenser yields a brownish-red fluorescent solution and an insoluble residue. This reddish fluorescent material is readily extractable by ether, chloroform, benzene, or xylene. It is probably an oxazone formed by hydrolysis of cresyl blue; and judging from its color changes it is a very much weaker base than cresyl blue.

The following experiments have a significant bearing on the question of the stability of cresyl blue as well as on the formation of oxazone and intermediate products.

Well dried samples of the dye were first shaken with anhydrous solvent to obtain evidence of the possible presence of red coloring matter. Following this, a small amount of distilled water was added and the extraction process repeated. For this purpose anhydrous ether, benzene, or xylene are well-suited since they do not dissolve appreciable amounts of cresyl blue chloride; chloroform is not as satisfactory because it dissolves large amounts of the latter also.

The results were very striking. Sample C and several similar products gave no perceptible coloration to the anhydrous solvents. Sample R gave a slight pink fluorescence. Sample A gave a more marked color. A specimen of the zinc chloride double salt which

we prepared gave a very marked red fluorescence. In other words, those samples which were prepared out of contact with water contained apparently no demonstrable quantities of the red coloring matter. Sample R (in which the effect of contact with water was kept at a minimum) showed the presence of only a slight amount; while the zinc chloride salt in which the action of water was not restricted showed apparently large amounts of the red substance present.

When water was added to the mixture of dry solvent and dye the result was the same for all solvents and for all samples apparently free from the red substance. Within about 10 minutes, there developed in the now aqueous solvent a pink fluorescence which became redder and more pronounced as contact of dye with water was prolonged. The preparations already contaminated with this material gave deeper red extracts.

It seems clear, therefore, that brilliant cresyl blue will remain "pure" only so long as it is kept out of contact with water. This point may be of but minor significance to the cytologist who has found empirically that solutions of definite composition and age yield the most desirable results in the staining of certain biological structures. It should, however, be of prime importance in physiological experiments where the integrity of the cresyl blue molecule is made the basis for experimentation and for theories of protoplasmic and membrane behavior.

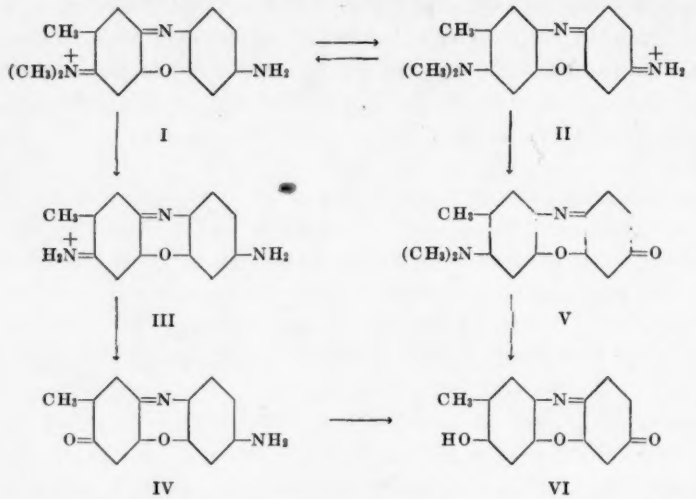
There is evidence that the red coloring matter from cresyl blue is not a single chemical entity. The fluorescent red material extracted by benzene or ether from refluxed aqueous dye is apparently a true oxazone. It is a very weak base and forms with acid a blue-violet salt which is insoluble in benzene or ether.

In addition there is extractable from aqueous solutions of the dye another substance which colors the solvent a fluorescent brown-red when extracted at pH 9 and 10, more pinkish when extracted at pH 8 and bluish-red with brown fluorescence at pH 6 and less. This material is evidently a stronger base than the above oxazone but much weaker than cresyl whose basic dissociation occurs near pH 10.7.

The base of cresyl blue is a brownish red substance formed above pH 10.7 and is extracted by benzene and ether, but the color imparted to these solvents is yellow to brown without the marked fluorescence of the two preceding substances.

Concerning the precise identity and properties of the hydrolysis products, which are assumed to be oxazone and intermediate prod-

ucts, there is little to be added at this time. Structurally, there are several possibilities, of which the following are the simplest.



The tautomerization I⇌II is a well recognized possibility. Stepwise oxidative demethylation, I→III, has been shown to occur readily in aqueous solutions of the analogous methylene blue series. The transformations, II→V and III→IV, to the corresponding oxazones is also readily accomplished with the splitting off of ammonia.

Until the products extracted from cresyl blue are analyzed chemically and carefully compared with these various structures it will not be possible to elucidate the perplexing series of decompositions occurring in aqueous solutions of the dye. The rate of formation has not been determined; but an idea of it can be gained from the fact that fresh solutions of cresyl blue contain only traces, solutions about 1 hour old contain 10 to 20 per cent, and solutions about 2 hours old may contain up to 50 per cent red coloring matter as determined by titration. Extremes of acidity and alkalinity favor its rapid production.

The red coloring matter which has been loosely termed "oxazone" in the present discussion may be a mixture of the several possible oxazones and various intermediate products of the interaction of water with cresyl blue. However, one of the red products seems to predominate to a large extent under the conditions of these experiments. It is a much weaker base than cresyl blue and forms a reversible oxidation-reduction system with its own reductant at a level of potential somewhat more electronegative than that of the cresyl blue : leuco-cresyl blue system; but the two systems do not overlap seriously and fairly satisfactory measurements can be obtained for cresyl blue.

There is another aspect of the electrometric data which may offer a clue to the organic chemist for unraveling the details of this complex but not unusual type of hydrolytic decomposition of the thiazines and oxazines. An inspection of the various titrations of cresyl blue and the Capri blues reveals evidence of the constant presence of small but definite amounts of an active impurity *electropositive* to the main system in addition to the more electronegative "oxazone." For the symmetrical and, therefore, more unstable Capri blues a correction has to be introduced. In the cresyl blue titrations its presence is evidenced by the negative trend of the potentials at the beginning of a reduction titration of the oxidant or at the end of an oxidation titration of the reductant. This is entirely apart from the occasional reoxidation of the leuco-dye by light or by oxygen leaks. Curiously enough, the proportion of this impurity seems to remain relatively low even in cases where the "oxazone" is permitted to mount to high values. Is this second impurity connected with the hydrolysis of the dye to oxazone? We suspect that it is, and that a process leading to structure IV or V, page 32, may be taking place. That is, there is first formed the demethylated product, which should be more unstable than the parent compound and therefore quickly go over to the oxazone. Thus the amount of dye would tend to decrease, the oxazone to increase, and the intermediate product to remain fairly constant but in small amount. It should be noted that usually no effect of this product was evident when fresh cresyl blue was reduced and then titrated with benzoquinone. The cause for this behavior is not clear; a small error in estimating the endpoint would account for this only partly.

ELECTRODE MEASUREMENTS

CONCENTRATION AND SALT EFFECTS

Having in mind the very marked variation of potential with concentration of Nile blue and of methylene blue (*cf.* Paper VIII), it was of interest to see whether this occurred with cresyl blue. The following simple experiment combined observations on a possible "salt effect" as well as on the concentration effect. Solutions of the compositions shown in Table 16 were prepared. The pH values of the buffers diluted with water were determined and considered the same as those of the buffers diluted with an equal volume of the partly reduced dye mixture. For a reference point, pH 1.008 was taken; and since the potentials in this region increase 0.0902 volt for each decrease of one unit of pH, the appropriate correction of the observed E_h values was applied to obtain the comparable values shown in the last column of Table 16. The

concentration effect appears to be negligibly small in solutions of about 0.2μ. When the ionic strength of the solution is increased to 0.3 to 0.6μ there occurs a +2 millivolt change in potential with two and threefold increases in concentration of the dye. The unpromising nature of the material made it seem unwise to pursue the matter in greater detail; and for the purpose of this investigation the effect of cresyl blue concentration has been rendered practically negligible by operating generally with solutions of the order of 0.00002 to 0.00005 molar.

It is not certain that there is an appreciable light effect on leucocresyl blue. To avoid complications from this source, the important measurements were made in a darkened north room.

TABLE 16.—*Cresyl blue—Effects of salt and dye concentrations on potentials*

Solution [1]	μ	pH	ΔpH $\times$0.0902	E_h	E_h corrected
50 c. c. C+ 5 c. c. BCB	0.64	1.008		0.4963	0.4963
50 c. c. C+10 c. c. BCB	.59	1.054	0.0041	.4940	.4981
50 c. c. C+15 c. c. BCB	.55	1.093	.0077	.4904	.4981
50 c. c. B+ 5 c. c. BCB	.36	1.047	.0035	.4929	.4964
50 c. c. B+10 c. c. BCB	.34	1.087	.0071	.4907	.4978
50 c. c. B+15 c. c. BCB	.32	1.126	.0106	.4875	.4981
50 c. c. A+ 5 c. c. BCB	.18	1.069	.0055	.4911	.4966
50 c. c. A+10 c. c. BCB	.17	1.107	.0089	.4873	.4972
50 c. c. A+15 c. c. BCB	.15	1.140	.0119	.4850	.4969

[1] Solution A=0.1 M HCl+0.1 M NaCl.
B=0.1 M HCl+0.3 M NaCl.
C=0.1 M HCl+0.6 M NaCl.
BCB=partially reduced, aqueous cresyl blue (sample R) approximately 0.0005 M before reduction.

ELECTROMETRIC TITRATIONS

A brief recital of a few of the preliminary titrations will indicate the nature of the difficulties encountered with this unstable system.

Sample C was dissolved in water and a considerable amount of sediment and insoluble greenish scum was filtered off. The filtrate was added to buffer No. 9, pH 4.846, to give a concentration of about 0.00025 M. The mixture was deoxygenated with purified nitrogen in the electrode vessel and titrated with reduced anthraquinone-β-sulphonate. The results are shown in the first part of Table 17. The potentials obtained after each addition of reducing agent adjusted themselves quickly, and the duplicate electrodes gave readings which agreed within 0.2 mv. A fairly sharp endpoint was reached at 11.5 c. c. of reducing agent, but the calculations for E'_0 on this basis give no constant value. When 1.5 c. c. is subtracted to allow for 13 per cent "oxazone" at the end of the titration, a semblance of order is obtained; and when, in addition, 0.6 c. c. is subtracted to allow for 4.3 per cent active contaminant at

the beginning of the titration, the E'_0 values reach the constancy shown in the second part of Table 17. The deviations at each end of the titration (bracketed values) are in the directions to be expected from the contaminating substances and are not due to sluggish electrodes or similar experimental conditions.

TABLE 17.—*Titration of cresyl blue chloride (sample C) with reduced anthraquinone-β-sulphonate in buffer No. 9, pH 4.846*

[Concentration of dye 0.00025 M]

Reducing agent c. c.	E_h	E'_0	Reducing agent corrected c. c.	E'_0 corrected
1	0.1940	0.1633	0.4	
2	.1812	.1608	1.4	(0.1584)
3	.1734	.1598	2.4	(.1594)
4	.1672	.1590	3.4	.1598
5	.1614	.1580	4.4	.1598
6	.1559	.1570	5.4	.1598
7	.1500	.1558	6.4	.1599
8	.1432	.1540	7.4	(.1603)
9	.1343	.1511	8.4	(.1621)
10	.1170	.1418	9.4	
11.5				
			Average=0.1598	

The titration is typical of those obtained from commercial samples of brilliant cresyl blue (vital stain). The "oxazone" found was formed during contact of the dye with water and was usually absent from the dry powders.

The occurrence of an insoluble residue in the dye is apparently not confined to the commercial product. For instance, the water-recrystallized sample A contained appreciable amounts of it. A solution of this specimen was made by grinding 0.044 gm. in 500 c. c. water. The sediment which was filtered off was found to be a dark granular material insoluble in water and slightly soluble in alcohol. It had none of the characteristic properties of cresyl blue. The dye solution was titrated with anthrahydroquinone-β-sulphonate in buffer No. 10, pH 5.317 (Table 18) and the measurements were continued beyond the endpoint for cresyl blue in order to reveal something of the more electronegative "oxazone" system. For the latter, the potentials were rather erratic, but the general level of the system (0.04 volt at pH 5.317) is fairly evident from the data in the second half of Table 18. This value was confirmed by other titrations at the same acidity.

The data show that there was also present about 5 per cent of active impurity at the start of the titration. When this correction is made, most of the divergent (bracketed) values line up around $E'_0 = +0.1276$.

TABLE 18.—*Titration of cresyl blue chloride (sample A) with anthrahydroquinone-β-sulphonate in buffer No. 10, pH 5.317*

[Concentration approx. 0.00003 M]

PART A: CRESYL BLUE SYSTEM

Reducing agent, c. c.	Reduction, per cent	$0.03006 \times \log \frac{[S_r]}{[S_o]}$	E_h	E'_o
1.0	10.42	−0.0281	0.1607	(0.1326)
1.5	15.63	−.0220	.1528	(.1308)
2.0	20.83	−.0174	.1472	(.1298)
2.5	26.04	−.0136	.1428	(.1292)
3.0	31.25	−.0103	.1391	(.1288)
3.5	36.46	−.0072	.1356	(.1284)
4.0	41.67	−.0044	.1326	(.1282)
4.5	46.88	−.0016	.1295	(.1279)
5.0	52.08	+.0011	.1266	.1277
5.5	57.29	.0038	.1237	.1275
6.0	62.50	.0067	.1207	.1274
6.5	67.71	.0097	.1176	.1273
7.0	72.92	.0129	.1142	.1271
7.5	78.13	.0167	.1106	.1273
8.0	83.33	.0210	.1062	.1272
8.5	88.54	.0267	.1012	(.1279)
9.0	93.75	.0353	.0943	(.1296)
9.6	100.00			
PART B: "OXAZONE" SYSTEM				
9.6	0			
10.0	20	−0.018	0.066	0.048
10.3	35	−.008	.056	.048
10.6	50	.000	.041	.041
11.0	70	+.011	.025	.036
11.3	85	+.023	.014	.037
11.6	100			

Another titration of sample A at pH 8.65 gave a similar set of data with $E'_o = -0.005$ volt. Following this, a rough preliminary determination was made of the variation of E'_o with pH, to see whether a consistent set of values was obtainable with material which was not only impure but also underwent decomposition while in solution. The results are shown in Table 19. The general agreement between observed and calculated values is rather poor, but the experiment indicated that with proper precautions a useful set of data could be obtained.

TABLE 19.—*Cresyl blue—Relation of E'_o to pH*

[Preliminary series, sample A]

Buffer No.	pH	E'_o observed	E'_o calculated[1]	Difference
1	1.07	+0.471	+0.486	−0.015
2	1.37	.457	.459	−.002
4	2.44	.366	.363	+.003
5	2.86	.325	.325	.000
6	3.27	.294	.288	+.006
8	4.35	.201	.196	+.005
9	4.85	.154	.160	−.006
10	5.38	.126	.124	+.002
13	6.08	.080	.085	−.005
15	6.80	.056	.054	+.002
16	7.40	.030	.034	−.004
20	8.08	+.008	+.012	−.004
22	8.65	−.005	−.005	.000
23	9.30	−.026	−.025	−.001
24	10.27	−.057	−.058	+.001
24½	10.79	−.075	−.079	+.004
27	11.82	−.134	−.134	.000
28	12.29	−.164	−.162	−.002

[1] See Table 24.

Electrode measurements of sample R.—This specimen obtained by salting out with NaCl contained about 41 per cent salt. It also contained a very small amount of the "oxazone." The following reduction titration illustrates the improvement in results.

The dye was dissolved in water (0.044 gm. in 500 c. c.) and filtered through asbestos. A small amount of insoluble residue was left behind. A 5 c. c. aliquot of this solution was mixed with 50 c. c. buffer of pH 1.995 and titrated with anthrahydroquinone-β-sulphonate dissolved in the same buffer. The titration was continued until the solution became colorless. No precipitate was detectable at the end. At this point a reverse titration was made and benzoquinone was added gradually. There first appeared a red coloration corresponding to reoxidation of the oxazone and after further addition of quinone the blue color of cresyl blue appeared. Table 20 gives the results. Part A shows the reduction of cresyl blue. The first three divergent (bracketed) values of E'_0 represent interference by the electropositive oxidizing impurity.

Part B, Table 20, gives the titration of the oxazone which was present to the extent of about 47 per cent of titratable material. The large proportion of oxazone was due primarily to the effect of the acid buffer on cresyl blue, and advantage was taken of this effect to obtain a better titration curve of the oxazone.

TABLE 20.—*Titration of cresyl blue chloride (sample R) with reduced anthraquinone-β-sulphonate in buffer No. 3, pH 1.995*

[Concentration approx. 0.00003 M]

PART A: CRESYL BLUE SYSTEM

Reducing agent, c. c.	Reduction, per cent	$0.03006\times\log\frac{[S_r]}{[S_o]}$	E_h	E'_0
1	9.17	−0.0299	0.4369	(0.4070)
2	18.35	−.0195	.4253	(.4058)
3	27.52	−.0127	.4182	(.4055)
4	36.70	−.0071	.4122	.4051
5	45.87	−.0022	.4071	.4049
6	55.04	+.0026	.4020	.4046
7	64.22	.0076	.3971	.4047
8	73.40	.0133	.3915	.4048
9	82.57	.0203	.3946	.4049
10	91.74	.0315	.3759	(.4074)
10.9	100.00			

PART B: "OXAZONE" SYSTEM

10.9	0			
12	11.58	−0.0265	0.338	(0.311)
13	22.11	−.0165	.310	(.293)
14	32.64	−.0095	.290	(.280)
15	43.17	−.0036	.278	.274
16	53.70	+.0019	.268	.270
17	64.23	.0071	.261	.268
18	74.76	.0142	.255	.269
19	85.29	.0229	.248	.271
20	95.82	.0409	.230	.271
20.4	100.00			

In another experiment at pH 1.995, leuco-cresyl blue was oxidized with benzoquinone, yielding a series of uniform E'_0 values, average 0.4044. The dye oxidant in this case was exposed for a shorter time to the action of the acid buffer and consequently the oxazone formed amounted to only 23.5 per cent of the total.

The titration of reduced cresyl blue with an oxidizing agent (benzoquinone) yields a smooth curve with reproducible potentials and also reveals the interfering impurities on each side of the cresyl blue system. The following experiment (with sample R) illustrates this and furnishes a useful datum for the final orientation of the cresyl blue system. The dye, 0.044 gm. was dissolved in 500 c. c. water, filtered through asbestos and quickly reduced with hydrogen in the presence of platinized asbestos. During the reduction the color changed first from blue to red, and then the red began to fade. The process was interrupted when the red color was almost but not entirely gone. The platinum black and asbestos were then filtered off. The resulting solution was faintly pink and contained a slight haze but no detectable precipitate. The hydrogen in the system was then displaced with deoxygenated nitrogen. Five c. c. of this stock solution were added to 50 c. c. of buffer No. 10, pH 5.317, previously deoxygenated, and the titration was made with a solution containing 0.375 mgm. benzoquinone in 50 c. c. buffer No. 10 +5 c. c. water.

TABLE 21.—*Titration of reduced cresyl blue (sample R) with benzoquinone in buffer No. 10, pH 5.317*

[Concentration by titration=0.00002 M]

Quinone, c. c.	Oxidation, per cent	$0.03006 \times \log \frac{[S_r]}{[S_o]}$	E_h	E'_0
3	3.29	+0.0442	+0.0888	(0.1330)
4	9.87	.0289	.0993	(.1282)
5	16.45	.0212	.1062	.1274
6.02	23.16	.0157	.1115	.1272
7	29.61	.0113	.1159	.1272
8	36.18	.0074	.1198	.1272
9	42.76	.0038	.1234	.1272
10	49.34	+.0003	.1270	.1273
10.98	55.79	−.0030	.1304	.1274
12.03	62.70	−.0068	.1342	.1274
13	69.08	−.0105	.1380	.1275
14.02	75.79	−.0149	.1426	.1277
15	82.24	−.0200	.1476	.1276
16	88.82	−.0270	.1543	.1273
17	95.39	−.0396	.1651	(.1255)
17.80	100.00			

Average=0.1274

The results are shown in Table 21. The first 2.5 c. c. of quinone were taken up by the electronegative oxazone system. The major portion of the quinone was consumed in the oxidation of the leuco-cresyl blue. The practically constant value for E'_0 (average +0.1274) indicates good agreement of the experimental data with the theoretical

curve. A fine white suspension, almost invisible, was found at the end of the experiment. In another titration at pH 5.317, the oxidant was titrated with reduced anthraquinone-β-sulphonate, yielding for E'_0 the average +0.1276.

A final orienting value for the E'_0 : pH curve was obtained at pH 8.644 by a titration of the reduced dye with benzoquinone. The reduction of the dye with hydrogen-platinum black was carried to a point where the red oxazone was converted entirely to the colorless state. Table 22 shows the results. The duplicate electrodes agreed well and the endpoint was sharp. The first 1.6 c. c. of quinone were consumed by the reduced oxazone, corresponding to 11.2 per cent of the total. The remainder was consumed by the leuco-cresyl blue, average $E'_0 = -0.0048$.

TABLE 22.—*Titration of reduced cresyl blue chloride with benzoquinone in buffer No. 22, pH 8.644*

[Concentration approx. 0.000025 M]

Quinone, c. c.	Oxidation, per cent	$0.03006 \times \log \frac{[S_r]}{[S_o]}$	E_h	E'_0
1.6	0.00			
4.0	18.97	+0.0190	−0.0233	−0.0043
5.0	26.88	.0131	−.0179	−.0048
6.0	34.78	.0082	−.0133	−.0051
7.0	42.69	+.0038	−.0088	−.0050
8.0	50.59	−.0003	−.0046	−.0049
9.0	58.50	−.0045	−.0004	−.0049
10.0	66.40	−.0089	+.0042	−.0047
11.0	74.31	−.0139	.0092	−.0047
12.0	82.21	−.0200	.0153	−.0047
13.0	90.12	−.0289	.0240	−.0049
14.25	100.00			

Average = −0.0048

The method of mixtures was also tried in buffer No. 22. The results are shown in Table 23. The various ratios of oxidized and reduced solutions gave a fairly orderly variation in E'_0 which can be accounted for by the interference of the more electronegative "oxazone" system. This can be corrected for by the data of the preceding titration in which it was found that the ratio of cresyl blue to oxazone was as 88.8 to 11.2. If this mixture is reduced and 1 c. c. is added to 4 c. c. of the unreduced mixture the two systems cresyl blue: leuco-cresyl blue and oxazone: leuco-oxazone will interact to a common potential. The resultant ratio of cresyl blue: leuco-cresyl blue can be determined approximately by assuming that the two systems are sufficiently separated on the potential scale so that the preponderant cresyl-blue system converts all the reduced oxazone to the oxidized form. In this process a corresponding amount of cresyl blue becomes reduced. Thus, in the above case the reduced oxazone acts upon the cresyl blue decreasing it to 3.552 − 0.112 = 3.44 c. c., and the leuco-cresyl blue is increased to 0.888 +

0.112 = 1.00 c. c. In the second part of Table 23 this correction is applied to the various mixtures, and the resulting corrected values for E'_0 line up in rough agreement with the average obtained in the preceding titration.

TABLE 23.—*Cresyl blue chloride (sample R)—Mixtures of oxidized and reduced solution in buffer No. 22, pH 8.644*

Oxidant, c. c.	Reductant, c. c.	E_h	E'_0	Cresyl blue corrected		E'_0 corrected
				Oxidant	Reductant	
4	1	+0.0149	−0.0020	3.44	1.00	−0.0030
3	2	−.0021	−.0065	2.44	2.00	−.0047
2.5	2.5	−.0071	−.0055	1.94	2.50	−.0038
2	3	−.0151	−.0097	1.44	3.00	−.0055
1	4	−.0336	−.0152	0.44	4.00	−.0048

Average = −0.0044

This analysis is incomplete in two details: One is the failure to allow for the increase in oxazone due to increased age of the dye solution; the other is failure to allow for the interference of the unidentified electropositive system also present. The rough agreement of the corrected E'_0 values is, therefore, perhaps partly fortuitous; but their general trend indicates that our interpretation is in the right direction.

RELATION OF E'_0 TO pH

For the final determination of the E'_0 : pH curve a fixed mixture of dye oxidant and reductant was made and 5 c. c. portions were added to 50 c. c. of various buffers. In preparing the fixed mixture, an excess of oxidant was introduced yielding a final solution the potential of which indicated the ratio cresyl blue: leuco-cresyl blue $= \frac{63}{37}$.

Under the conditions a smaller proportion of oxidant would have been more desirable; for it would have favored a smaller conversion to oxazone; but this improvement was not made because the data obtained with the above mixture seem sufficient for present purposes.

In the acid buffers Nos. 1 to 7, the electrode potentials remained constant or drifted positively to plateau values which remained constant for over 30 minutes; in the next three, the values drifted positively to a peak and then drifted negatively. In all of these cases the peak or plateau values were chosen. Negative drifts in potentials occurred from pH 4.4 to 12.3. In buffers Nos. 8 to 22 the electrode potentials drifted to peak negative values within about 5 minutes and then turned more and more positive. These negative peaks were selected as the probable equilibrium values. In the remaining alkaline solutions, Nos. 23 to 28 the potentials went negative, slowly at first

and then rapidly. The speed of this drift in potential appeared to increase with the alkalinity. In these cases the first obtainable readings were chosen.

When the electrode vessels were opened for cleaning, all solutions excepting No. 1 contained a slight amount of a fine white precipitate.

In Table 24 will be found the data obtained. The experimental data (circles) and theoretical curve are also shown in Figure 3. The agreement between the observed and calculated values for E'_o is comparatively good. There is a negative deviation in the acid buffers Nos. 1 and 2 which may be due to the effect of the second potentially basic group in the oxidant (see Dissociations). The predominance of negative deviations in the alkaline region is an expression of the experimental fact that rapid negative drifts occurred during the measurements and the initial potentials taken within one minute were uncorrected for this drift.

TABLE 24.—*Cresyl blue—Relation of E'_o to pH (Final series; sample R)*

[Concentration approx. 0.000025 M]

Buffer No.	pH	E'_o observed	E'_o calculated [1]	Difference
1	1.076	+0.480	0.486	−0.006
2	1.363	.453	.460	−.007
3	1.995	.403	.403	.000
4	2.454	.361	.361	.000
5	2.879	.325	.323	+.002
6	3.421	.277	.275	+.002
7	3.860	.237	.237	.000
8	4.413	.195	.191	+.004
9	4.898	.157	.156	+.001
10	5.397	.123	.123	.000
13	5.870	.097	.096	+.001
14	6.258	.076	.076	.000
15	6.737	.055	.057	−.002
16	7.424	.033	.033	.000
17	7.849	.019	.019	.000
21	8.411	+.001	.002	−.001
22	8.644	−.004	−.005	+.001
23	9.261	−.029	−.024	−.005
23½	9.588	−.035	−.034	−.001
24	10.18	−.059	−.054	−.005
24½	11.02	−.092	−.090	−.002
26	11.47	−.115	−.114	−.001
27	11.77	−.131	−.131	.000
28	12.27	−.163	−.160	−.003

[1] Calculated by means of equation (12) using

$E'_{pHO}=+0.5825$ $K_{r3}=2.512\times10^{-3}$ $pK_{r2}=6.30$
$K_{ob}=1.035\times10^{-3}$ $K_w=1.88\times10^{-14}$ $pK_{r3}=4.60$
$K_{r2}=5.012\times10^{-7}$ $pK_{ob}=2.985$ $pK_w=13.725$

ELECTRODE EQUATION

The electrode equation (11) for Nile blue applies also to the case of the cresyl blue system. When $[S_r]=[S_o]$,

$$E_h=E_o-0.03006\log([H^+]K_{ob}+K_w)+0.03006\log(K_{r2}K_{r3}[H^+]^2+K_{r3}[H^+]^3+[H^+]^4) \quad (12)$$

For the derivation, see p. 26.

DISSOCIATIONS

As in the case of Nile blue, the determination of the dissociation constants was made from a graphical analysis of the E'_o: pH curve of the system. It should be noted that the precision of these values

is primarily dependent on the precision of the points on the curve as established by the electrode measurements. In the case of cresyl blue, considerable drifts in potential in the alkaline region introduce a more or less serious factor of uncertainty in the position of the terminal 0.06-slope and, therefore, of the value for the dissociation constant of the oxidant. The unsatisfactory nature of the dye made unattractive any attempt at determination of the basic constant by some independent method. Its general order of magnitude is well established by the present data to within one or two units in the first decimal place.

The allocation of dissociation constants follows the plan already outlined. The E'_o : pH curve (Fig. 3) has three distinct bends representing detectable dissociations within the range of pH studied. Analysis of the curve places the dissociation of the relatively strong polar group of the oxidant at pH 10.74. That is

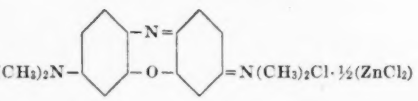
$$pK_{ob} = pK_w - pH = 13.725 - 10.74 = 2.985.$$

This value happens to yield the best fit to the experimental curve. As indicated above, the value $pK_{ob} = 3.0$ is all that is justified by the data. The strength of the polar oxidant as a base is approximately the same as that of dimethylamine.

The dissociation of the nonpolar amino group in cresyl blue is too low to appear between pH 1 and 12. However, a definite color change from blue to pink-violet occurs as the acidity is increased from 0.2 N to 2 N HCl.

The two remaining dissociations indicated on the E'_o:pH curve belong to the reductant: The one occurring at pH 4.6 is due to the simple amino group, $pK_{r2} = 4.6$, and the one at pH 6.3 to the dimethylamino group, $pK_{r3} = 6.3$.

Methyl Capri Blue

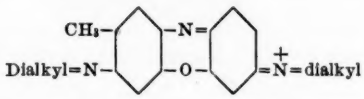


Tetramethyl diaminophenoxazine chloride-zinc chloride double salt

This oxazine analogue of methylene blue was studied primarily with the object of obtaining a comparison of the oxidation-reduction characteristics of the two classes of dyes represented by analogous structures. Apart from its intrinsic interest, this comparison may be of importance in the general question of the effects of substitution on the properties of organic compounds.

The Capri blue dyes were first reported by Bender in British patent No. 13565 (1890). Symmetrical tetramethyl Capri blue was made from *m*-dimethylaminophenol and *p*-nitrosodimethylaniline. The

difficulties in preparation and the low yields have apparently stood in the way of its commercial exploitation, and the more easily obtainable toluphenazine derivatives are listed as Capri blue GN, GON or V in the various editions of Schultz's *Farbstofftabellen*. These compounds consist of certain combinations of dialkylamines in the structure:



Rowe's *Colour Index* lists the above structures as No. 876: Brilliant Blue CC and Capri Blue GON. A note states than Capri Blue GN is made from dimethyl-*m*-aminophenol and *p*-nitrosodimethylaniline. This would yield the oxazine analogue of methylene blue which is the subject of the present investigation.

In this paper, we shall refer to the symmetrical tetramethyl phenoxazine as methyl Capri blue.

SYNTHESIS, PURIFICATION, ANALYSIS, AND PROPERTIES

In 1895, Möhlau and Uhlmann reported that the chloride of methyl Capri blue was too soluble to isolate and obtained a small amount of the iodide after some difficulty with poor yields. The fact that solutions of their product were blue with a pronounced brown-red fluorescence suggests that they were dealing with contaminated material. Kehrmann and Poplawski (1909) described the preparation of the nitrate of Capri blue and later, Kehrmann, Havas and Grandmougin (1914) reported the isolation of the chloride. Subsequent papers by Kehrmann and his associates described the preparation of the compound by various methods but details regarding yields are not satisfactory.

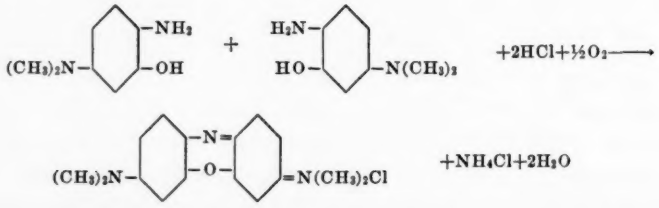
In the following synthesis, the method of Kehrmann and Poplawski was employed, except that the final product was isolated as the zinc chloride double salt instead of the nitrate. It was anticipated that presence of the nitrate group would seriously interfere with the oxidation-reduction measurements. Subsequent events, however (see section on ethyl Capri blue) have indicated that the nitrate anion apparently does not interfere with the electrode measurements.

The product was obtained by the air oxidation of *asym.* dimethyloxy-*p*-phenylene diamine (1-hydroxy-3-dimethylamino-6-aminobenzene). The intermediate was prepared as follows from *m*-dimethylaminophenol. One hundred grams of the latter were dissolved in 300 grams of 32 per cent HCl and chilled to below 0° C.; and there was slowly added with stirring a chilled solution of 33 grams $NaNO_2$ in 90 c. c. water. The temperature was not allowed to rise above 0° C. The yellow nitroso compound crystallized out and was filtered almost dry.

The nitroso compound was then ground up with 100 c. c. cold concentrated HCl in a chilled mortar and added slowly to a chilled solution of 190 grams $SnCl_2 \cdot 2H_2O$ in 150 c. c. concentrated HCl. The mixture was cooled to below 0° C. and the crystallized tin double salt removed by filtration. An equal amount of concentrated HCl was added to the filtrate and an additional quantity of the product separated out on chilling.

The tin double salt was then dissolved in 5 liters of water, and H_2S was passed through the solution which was shaken mechanically. The sulphides were then filtered off, and H_2S was removed from the filtrate by an air stream.

The solution of *asym.* dimethyloxy-*p*-phenylene diamine was heated on a boiling water bath. Sodium acetate was added until the pH of the solution was between 3 and 4 (the dye forms quickly at this pH); and a stream of air was bubbled through. Aeration was continued until color production reached a maximum. The reaction may be indicated as follows:



The crude chloride of methyl Capri blue in acetate buffer was concentrated to 1 liter by evaporation and filtered. The residue was heated with 1,000 c. c. water and filtered off, the process being repeated three times. The filtrates were combined, acidified with HCl, zinc chloride added, and the product salted out with NaCl. The zinc chloride double salt of the dye was obtained. This was redissolved, filtered, and recrystallized with a minimum of zinc chloride. The crystals were washed with a saturated solution of NaCl acidified with HCl and then dried in a desiccator over stick NaOH. The product was finally extracted with benzene to remove red coloring matter.

The product contained admixed sodium chloride. The purity of the compound was therefore estimated from the chloride determination and the zinc : nitrogen ratio. The dry zinc salt contained very little coloring matter extractable by xylene. Analysis yielded 6.61 per cent zinc, 8.62 per cent nitrogen, and 28.62 per cent chlorine; calculated for $C_{16}H_{18}ON_3Cl \cdot \frac{1}{2}\ (ZnCl_2)$: 8.79 per cent Zn, 11.30 per cent N, and 19.08 per cent Cl. Zn : N ratio = 0.778; found 0.767. The 15.26 per cent excess chlorine corresponds to 25.16 per cent admixed NaCl; and the nitrogen content is equivalent to 70 per cent dye. This accounts for 95.2 per cent of the total.

Methyl Capri blue salt is readily soluble in water giving a deep-blue solution of high tinctorial power. This color shows no immediate change on the addition of dilute acid or alkali. The dye colors buffer solutions from pH 1 to 10 a uniform blue. After standing a while in the extreme alkaline buffers and especially in normal NaOH, the compound appears to undergo a change and the blue color is modified to a purple which fades slowly.

A little red coloring matter is extractable with xylene from solutions in buffers pH 1 to 8; much more is extracted from the alkaline solutions.

This compound resembles methylene blue in that its leuco-derivative in aqueous solution is sensitive to light. A short exposure to diffuse daylight results in a marked blue coloring of the solution. To avoid difficulties from this source, most of the measurements were made at night in protected apparatus and in a darkened room.

The reductant is also much less soluble than the oxidant. Therefore, operations were carried out in very dilute solutions.

ELECTRODE MEASUREMENTS

SALT AND CONCENTRATION EFFECTS

Salt and concentration effects were examined as in the preceding section, the object being to reveal the general nature and magnitude of the phenomena. Solutions of the compositions shown in Table 25 were prepared and their pH values determined. Effects on pH of the minute amounts of added dye were ignored; the pH values of the buffers diluted with water were considered the same as those of the buffers diluted with the partly reduced dye solution. The solution with pH 1.008 was taken as a reference point, and the E_h of the others was corrected to give a comparable series of values. Table 25 shows an appreciable salt effect; an increase from 0.18 to 0.64 μ corresponds to about 4 mv. increase in potential. There is also present a concentration effect which is of a lower order. A threefold increase in concentration produced a *decrease* in potential of 1 to 1.5 mv.

TABLE 25.—*Methyl Capri blue—Effects of salt and dye concentration on potentials*

Solution [1]	μ	pH	ΔpH $\times$0.0902	E_h	E_h corrected
50 c. c. C+ 5 c. c. Capri	0.64	1.008		0.3870	0.3870
50 c. c. C+10 c. c. Capri	.59	1.054	0.0041	.3827	.3868
50 c. c. C+15 c. c. Capri	.55	1.093	.0077	.3779	.3856
50 c. c. B+ 5 c. c. Capri	.36	1.047	.0035	.3817	.3852
50 c. c. B+10 c. c. Capri	.34	1.087	.0071	.3770	.3841
50 c. c. B+15 c. c. Capri	.32	1.126	.0106	.3731	.3837
50 c. c. A+ 5 c. c. Capri	.18	1.069	.0055	.3774	.3829
50 c. c. A+10 c. c. Capri	.17	1.107	.0089	.3752	.3841
50 c. c. A+15 c. c. Capri	.15	1.140	.0119	.3700	.3819

[1] Solution A=0.1 M HCl+0.1 M NaCl.
B=0.1 M HCl+0.3 M NaCl.
C=0.1 M HCl+0.6 M NaCl.
Capri=partially reduced, aqueous methyl Capri blue chloride-zinc chloride approximately 0.0002 M before reduction.

ELECTROMETRIC TITRATIONS

Methyl Capri blue-zinc chloride was dissolved in water to give a 0.0002 M solution, and 5 c. c. of this were titrated in buffer No. 6, pH 3.329 with reduced anthraquinone-β-sulphonate. The duplicate agreeing electrodes reached stable potentials quickly and a smooth titration curve was obtained. At the end of the titration, there occurred a slight overlap of the anthraquinone system, and the graphic estimation of the endpoint from the curve involves some uncertainty. When the electrode vessel was opened a fine white precipitate was observed.

The results are shown in Table 26. The first part of this table shows the E'_0 values calculated on the basis of the estimated endpoint. There is a progressive deviation such as would be caused by interference of a foreign active substance at the *start* of the titration. If it be assumed that the first 2 c. c. of reducing agent were consumed by this foreign oxidant, there is obtained a fairly uniform set of corrected values for E'_0 (average = 0.1780 at pH 3.329) over the significant portion of the curve. The deviating (bracketed) values at the end of the titration can not be easily interpreted because the exact endpoint is somewhat in doubt and overlap of the anthraquinone-β-sulphonate system obscures the effect of possible active impurity in the same region.

TABLE 26.—*Titration of methyl Capri blue-zinc chloride double salt with anthrahydroquinone-β-sulphonate in buffer No. 6, pH 3.329*

[Dye concentration 0.00002 M]

Reducing agent, c. c.	E_h	E'_0	Reducing agent corrected c. c.	E'_0 corrected
2	+0.2342	+0.2041	0	
3	.2138	.1896	1	(+0.1753)
4	.2061	.1864	2	(.1774)
5	.2005	.1845	3	.1778
6	.1961	.1832	4	.1779
7	.1924	.1824	5	.1780
8	.1892	.1818	6	.1780
9	.1863	.1814	7	.1781
10	.1834	.1809	8	.1780
11	.1808	.1807	9	.1781
12	.1782	.1805	10	.1781
13	.1755	.1802	11	.1780
14	.1728	.1800	12	.1779
15	.1701	.1799	13	.1780
16	.1671	.1797	14	.1779
17	.1641	.1799	15	.1782
18	.1607	.1801	16	(.1785)
19	.1568	.1806	17	(.1790)
20	.1523	.1819	18	(.1803)
21	.1468	.1856	19	(.1848)
22.1			20.1	

Average = +0.1780

Another titration at pH 3.329 was made, this time using an unpurified specimen of methyl Capri blue *chloride*. It contained about 40 per cent active impurity at the start of the reduction titration. A

rough estimate of the E'_0 of this impurity places it at +0.34 volt. The methyl Capri blue system was again found to be at +0.178 volt.

To confirm the occurrence of active impurity on the electronegative side of the Capri blue system, the dye was reduced and titrated at pH 4.911 with benzoquinone. The oxidation-reduction titration proceeded smoothly, yielding prompt equilibrium potentials and a sharp endpoint at 15.00 c. c. quinone. Calculation on this basis yields a discordant set of E'_0 values (see first part of Table 27). The deviations are such as would be caused by interferences at both ends of the titration. When 0.6 c. c. is subtracted from the experimental endpoint there is obtained the uniform set of values (average $E'_0 = 0.0467$) of the second half of Table 27. The divergent (bracketed) values at the top of the table indicate the interference of a small amount of active impurity at the electronegative side of the Capri blue system.

As would be expected, larger proportions of the two impurities were found in a quinone titration at pH 1.988. The mid-portion of the titration curve yielded a concordant set of E'_0 values, average 0.2970, after application of corrections.

TABLE 27.—*Titration of reduced methyl Capri blue-zinc chloride with benzoquinone in buffer No. 9, pH 4.911*

[Dye concentration 0.00002 M]

Quinone, c. c.	E_h	E'_0	Quinone corrected c. c.	E'_0 corrected
1	+0.0163	+0.0508	1	(+0.0502)
2	.0237	.0482	2	(.0475)
3	.0295	.0476	3	.0469
4	.0343	.0475	4	.0468
5	.0385	.0477	5	.0467
6.01	.0423	.0476	6.01	.0467
7	.0459	.0476	7	.0466
8	.0496	.0479	8	.0467
9	.0533	.0480	9	.0466
10	.0575	.0484	10	.0468
11	.0621	.0489	11	.0468
12	.0678	.0497	12	.0468
13	.0757	.0512	13	.0466
14	.0905	.0561	14	(.0441)
15			14.4	

Average = 0.0467

RELATION OF E'_0 TO pH

A stock aqueous solution was made of the zinc chloride double salt and a portion of it was reduced with hydrogen in the presence of platinum black. The filtered reductant solution was water-white with a faint lavender tint in thick layers. The reductant was added to deoxygenated oxidant to give a mixture of fixed ratio of the two components; and 5 c. c. of the mixture were measured in each of the buffers as shown in Table 28 to determine the variation of potential with pH. The potential readings behaved differently in the different

buffers and pH regions. In buffers Nos. 1 and 3 there occurred a negative drift to a peak value followed by a positive drift. In buffers Nos. 5 to 10½ there occurred a slow positive drift and the first values were selected. Constant values lasting at least five minutes were obtained in buffers Nos. 13 to 16. In buffers Nos. 17 to 22, the potentials drifted to a negative peak within about two minutes and then became progressively more positive. In the more alkaline buffers there occurred rapid negative drifts and the first readings were selected. The negative deviations from the calculated values in this region (see Table 28) are no doubt due to this drift. Corrections by extrapolation to zero time were not made. The relation of E'_o to pH is shown in Table 28 and in Figure 3.

TABLE 28.—*Methyl Capri blue—Relation of E'_o to pH*

[Total concentration, 0.00002 M]

Buffer No.	pH	E'_o found	E'_o calculated [1]	Deviation
1	1.076	+0.380	0.380	0.000
3	1.988	.297	.298	−.001
5	2.879	.218	.218	.000
6	3.329	.177	.177	.000
8	4.419	.085	.083	+.002
9	4.911	.047	.045	+.002
9½	5.136	.029	.029	.000
10	5.397	+.011	+.012	−.001
10½	5.710	−.008	−.006	−.002
13	5.870	−.017	−.015	−.002
13½	6.081	−.026	−.025	−.001
14	6.258	−.033	−.033	.000
14½	6.515	−.044	−.044	.000
15	6.731	−.052	−.052	.000
15½	7.052	−.063	−.063	.000
16	7.426	−.074	−.075	+.001
17	7.789	−.086	−.086	.000
20	8.038	−.094	−.094	.000
21	8.382	−.104	−.104	.000
22	8.649	−.110	−.112	+.002
23	9.248	−.131	−.130	−.001
24	10.10	−.156	−.156	.000
24½	10.91	−.184	−.180	−.004
26	11.44	−.198	−.196	−.002
28	12.27	−.225	−.221	−.004

[1] Calculated by equation (14) using—

$E'_{pH\ o}=0.477$ $pK_{r2}=6.10$ $pK_{r3}=4.85$ $K_w=1.88\times10^{-14}$ $K_{r2}=7.943\times10^{-7}$ $K_{r3}=1.413\times10^{-5}$

ELECTRODE EQUATION AND DISSOCIATIONS

The general equation (10) for the oxazines is applicable to the Capri blue system. Since dissociation of the oxidant does not occur within the pH range studied, the general equation may be simplified by the elimination of K_{ob} from the last term. Rewrite equation (10) as follows:

$$E_h=C-0.03006\log\left[\frac{[S_r]}{[S_o]}\times\frac{K_{r1}K_{r2}K_{r3}K_{ob}[H^+]}{K_{ob}[H^+]}\times\frac{1}{K_{r1}K_{r2}K_{r3}+K_{r2}K_{r3}[H^+]+K_{r3}[H^+]^2+[H^+]^3}\right]\qquad(13)$$

Since K_{ob} is considered to be very large, K_w drops out from (10) as negligibly small. Canceling, and gathering constants under E_o

(K_{r1}, it will be recalled is negligibly small), there is obtained the electrode equation for Capri blue, viz:

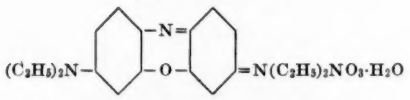
$$E_h = E_o - 0.03006 \log \frac{[S_r]}{[S_o]} + 0.03006 \log (K_{r2}K_{r3}[H^+] + K_{r3}[H^+]^2 + [H^+]^3) \quad \text{------(14)}$$

Dissociations.—The E'_o : pH curve (Fig. 3) confirms the observation that no dissociation of the oxidant of methyl Capri blue occurs within the pH range studied. That is, the polar terminal nitrogen of the dimethylamino group is a very strong base, and K_{ob} is too large to be suppressed by the alkaline buffers employed.

The curve has two bends the directions of which indicate dissociations in the reductant. The values of the corresponding dissociation constants are estimated graphically as:

$$pK_{r2} = 6.10;\ K_{r2} = 7.94 \times 10^{-7}$$
$$pK_{r3} = 4.85;\ K_{r3} = 1.41 \times 10^{-5}$$

Ethyl Capri Blue



Tetraethyldiaminophenoxazine nitrate

The examination of the tetraethyl homologue of methyl Capri blue offers an opportunity for obtaining additional data on the effects of substitution on the physico-chemical properties of organic systems. Although the Capri blues were found not to be ideal systems for precise quantitative comparisons, they revealed quite clearly the general magnitude and direction of these effects.

The first report, outside of the patent literature, on the symmetrical tetraethyl phenoxazine was made by Möhlau and Uhlmann (1895) who prepared the iodide after considerable difficulty. The prospects of better yields led us to the synthesis of the zinc chloride double salt and the nitrate.

SYNTHESIS, PURIFICATION, ANALYSIS, AND PROPERTIES

Preparation of ethyl Capri blue.—The method followed was similar to that for the methyl homologue. The starting material was *m*-diethylaminophenol (Eastman). This was converted to the nitroso derivative which was reduced to the *asym.* diethyloxy-*p*-phenylene diamine (1-hydroxy-3-diethylamino-6-aminobenzene). The solution of diamine was heated on a water bath, solid sodium acetate and sodium carbonate were added, and air was passed through until maximum color production occurred. A considerable amount of blue-black tarry substance separated out. The tarry material was extracted with hot water several times and the filtrates were com-

bined. Part of the dye was converted to the zinc double salt and part to the nitrate.

Ethyl Capri blue-zinc chloride.—The filtered dye solution was acidified with HCl and zinc chloride added. The crude zinc double salt was then salted out with NaCl. After filtration and drying, the product was extracted with anhydrous ether which removed a red substance. The main product was redissolved in water and reprecipitated with $ZnCl_2$ and NaCl three times. The crystals of the zinc double salt were then washed with acidified saturated NaCl solution. The yield was estimated at less than 10 per cent.

Ethyl Capri blue nitrate.—A solution of sodium nitrate was added to the filtered crude dye solution. After standing overnight in the cold the crystals of ethyl Capri blue nitrate were filtered off. The nitrate was redissolved in hot water, filtered, and cooled. Sodium nitrate was then added to give a final concentration of 50 gm. per 100 c. c., the crystals of ethyl Capri blue nitrate which separated out were washed with saturated $NaNO_3$ solution and dried. The yield was estimated at less than 10 per cent.

Analyses.—The zinc double salt yielded on analysis 9.37 per cent nitrogen and 7.13 per cent zinc; calculated, 9.82 per cent N, 7.64 per cent Zn. This corresponds to about 95 per cent purity. The sodium chloride content was not determined, but it probably accounts for the major portion of the remainder.

The cystalline nitrate of ethyl Capri blue contained admixed sodium nitrate. The results of the analyses are as follows: Sodium, 4.07 per cent (dry basis), corresponding to 15.02 per cent admixed $NaNO_3$, purity 85 per cent; nitrogen, calculated, 14.51 per cent, found 14.84 per cent. If the nitrogen from admixed sodium nitrate be subtracted, there is obtained 12.36 per cent N, or 85.2 purity. Carbon, calculated, 62.14 per cent, found 53.74 per cent, or 86.5 per cent purity. All the analyses were made on well-mixed desiccator-dried specimens. Heat was avoided to prevent possible decomposition.

Moisture found by heating *in vacuo* at 110° for 24 hours was 3.89 per cent, which corresponds closely to 85+% of 4.46 calculated for 1 mole water of crystallization.

Properties.—Aqueous solutions of ethyl Capri blue have a greenish tint as compared with those of the methyl homologue. In other respects the properties of the two compounds are similar.

ELECTRODE MEASUREMENTS

SALT AND CONCENTRATION EFFECTS

Salt and concentration effects were examined as in the case of the methyl homologue. The results are shown in Table 29. There is a −1 mv. change in potential for a threefold increase in concentration at the pH of the experiment. There is also evident a more appre-

ciable change in the opposite direction as the ionic strength of the solution is increased; that is, increase from 0.15 to 0.55 μ is associated with a +5 mv. change in potential.

TABLE 29.—*Ethyl Capri blue—Effects of salt and dye concentrations on potentials*

Solution [1]	μ	pH	ΔpH ×0.0902	E_h	E_h corrected
50 c. c. C+5 c. c. ECB	0.64	1.008		0.4644	0.4644
50 c. c. C+10 c. c. ECB	.59	1.054	0.0041	.4600	.4641
50 c. c. C+15 c. c. ECB	.55	1.093	.0077	.4557	.4634
50 c. c. B+5 c. c. ECB	.36	1.047	.0035	.4584	.4619
50 c. c. B+10 c. c. ECB	.34	1.087	.0071	.4544	.4615
50 c. c. B+15 c. c. ECB	.32	1.126	.0106	.4503	.4609
50 c. c. A+5 c. c. ECB	.18	1.069	.0055	.4540	.4595
50 c. c. A+10 c. c. ECB	.17	1.107	.0089	.4500	.4589
50 c. c. A+15 c. c. ECB	.15	1.140	.0119	.4465	.4584

[1] Solution A=0.1 M HCl+0.1 M NaCl
B=0.1 M HCl+0.3 M NaCl
C=0.1 M HCl+0.6 M NaCl
ECB=partially reduced aqueous ethyl Capri blue-zinc chloride, approximately 0.00028 M before reduction.

ELECTROMETRIC TITRATIONS OF THE ZINC SALT

A stock solution of the dye was made by dissolving 0.0236 gm. in 200 c. c. water; and a 5 c. c. aliquot was titrated with reduced anthraquinone-β-sulphonate at pH 3.329. A smooth titration curve was obtained with an endpoint at 32 c. c. reducing agent. A fine white precipitate was found when the electrode vessel was opened. The calculation of E'_0 from the experimental data gave the progressive set of values seen in the first portion of Table 30. When correction (*A*-correction) was applied for interference by about 19 per cent of active impurity (presumably oxazone) there was obtained the more orderly series of values of the second part of Table 30; and when allowance (*B*-correction) was also made for the equivalent of 1 c. c. (3 per cent) of active impurity at the start of the titration, the uniform series of the last part of Table 30 was obtained (average $E'_0=0.2384$ at pH 3.329).

TABLE 30.—*Titration of ethyl Capri blue-zinc chloride with reduced anthraquinone-β-sulphonate in buffer No. 6, pH 3.329*

[Total dye concentration at start 0.000025 M]

Reducing agent c. c.	E_h	E'_0	Red. agent *A*-corr.	E'_0 *A*-corr.	Red. agent *B*-corr.	E'_0 *B*-corr.
2	+0.2791	+0.2438	2	+0.2466	1	(+0.2375)
3	.2699	.2403	3	.2432	2	(.2379)
4	.2643	.2389	4	.2419	3	(.2381)
5	.2600	.2380	5	.2411	4	.2382
6	.2565	.2374	6	.2407	5	.2383
7	.2536	.2369	7	.2404	6	.2384
8	.2510	.2367	8	.2403	7	.2385
9	.2484	.2361	9	.2399	8	.2384
10	.2462	.2359	10	.2399	9	.2385
11	.2439	.2355	11	.2397	10	.2384
12	.2417	.2350	12	.2395	11	.2384
13	.2396	.2347	13	.2394	12	.2384
14	.2375	.2342	14	.2393	13	.2383
15	.2354	.2338	15	.2392	14	.2383
16	.2333	.2333	16	.2392	15	.2383
17	.2310	.2326	17	.2390	16	.2382
18	.2287	.2320	18	.2390	17	.2382
19	.2263	.2312	19	.2390	18	.2383
20	.2237	.2304	20	.2390	19	.2383
21	.2210	.2294	21	.2392	20	(.2386)
22	.2179	.2282	22	.2395	21	(.2389)
23	.2144	.2267	23	.2402	22	(.2396)
24	.2103	.2246	24	.2415	23	(.2409)
25	.2060	.2237	25	.2457	24	(.2451)
26	.2012	.2203	26.2		25.2	
28	.1897	.2151				
32						

* Average=0.2384

NOTE.—*A*-corr.=correction for active impurity at end of titration. *B*-corr.=*A*-corr.+correction for impurity at start.

Another reduction titration with fresh solutions was made at pH 4.901, and a sharp endpoint was reached at 24.9 c. c. anthrahydroquinone. When the electrode vessel was opened there was found a very fine, almost imperceptible white precipitate; the solution was colorless. Calculation from the experimental data yielded progressively varying E'_0 values such as would be caused by interference of active impurities at each end of the main titration curve. Application of the *B*-correction gave the uniform values seen in the second part of Table 31, average $E'_0=0.0996$ at pH 4.901.

TABLE 31.—*Titration of ethyl Capri blue-zinc chloride with reduced anthraquinone-β-sulphonate in buffer No. 9, pH 4.901*

Reducing agent c. c.	E_h	E'_o	Reducing agent (*B*-corr.)	E'_o (*B*-corr.)
2	0.1313	0.0994	1.6	(0.0980)
3	.1250	.0990	2.6	.0997
4	.1200	.0984	3.6	.0997
5	.1160	.0980	4.6	.0997
6	.1125	.0975	5.6	.0996
7	.1095	.0972	6.6	.0997
8	.1067	.0969	7.6	.0997
9	.1039	.0965	8.6	.0995
10	.1014	.0962	9.6	.0996
11	.0988	.0957	10.6	.0996
12	.0962	.0953	11.6	.0995
13	.0936	.0948	12.6	.0995
14	.0909	.0942	13.6	.0996
15	.0881	.0935	14.6	.0997
16	.0850	.0927	15.6	.0999
17	.0817	.0917	16.6	(.1003)
18	.0780	.0905	17.6	(.1011)
19	.0739	.0891	18.6	(.1030)
20	.0690	.0874	19.6	(.1078)
21	.0634	.0854	20.6	
22	.0565	.0830		
23	.0478	.0803	Average=0.0996	
24.9				

To check the last determination an oxidation titration was made using ferricyanide in the same buffer. A sharp endpoint was obtained. No precipitate was detectable in the colorless solution when the electrode vessel was opened. The results are shown in Table 32. Application of correction for interference at the start of the titration yields the average $E'_o = 0.0997$. The proportion of interfering impurity at the end was too small to make itself evident in this case.

A similar titration with benzoquinone at the same pH gave $E'_o = 0.0998$ after application of the above corrections.

TABLE 32.—*Titration of reduced ethyl Capri blue-zinc chloride with ferricyanide in buffer No. 9, pH 4.901*

$K_3Fe(CN)_6$ c. c.	E_h	E'_o	$K_3Fe(CN)_6$ (corrected) c. c.	E'_o (corrected)
1	0.0638	0.0944	0.2	
2	.0744	.0946	1.2	(0.1013)
3	.0824	.0958	2.2	(.0999)
4	.0887	.0967	3.2	.0996
5	.0941	.0973	4.2	.0996
6	.0992	.0978	5.2	.0997
7	.1043	.0982	6.2	.0998
8	.1096	.0984	7.2	.0998
9	.1159	.0986	8.2	.0998
10	.1244	.0987	9.2	.0998
11.4			10.6	
			Average=0.0997	

In order to obtain additional points for the more precise placement of the E'_o : pH curve two more reduction titrations were made of the zinc salt. These will be cited without tabulation of the experimental data. One made at pH 5.317 gave the average $E'_o = 0.0619$, and the other at pH 5.446 gave $E'_o = 0.0490$ after application of the usual corrections.

TITRATIONS OF ETHYL CAPRI BLUE NITRATE

The suspicion that the potentially reactive NO_3 ion would interfere with the oxidation-reduction measurements induced us to neglect the electrometric examination of this beautifully crystalline salt of ethyl Capri blue until after completion of the measurements on the zinc salt. It was then found that equally good titrations could be obtained on the nitrate. Several of these will be presented for illustration and comparison.

A solution of the nitrate was titrated with reduced anthraquinone-β-sulfonate at pH 4.901. Prompt equilibrium potentials were obtained after each addition of reducing agent, and a sharp endpoint at 20 c. c. A fine, white, granular precipitate was found in the colorless solution when the electrode vessel was opened. Table 33 shows the results obtained. Application of corrections (*B*-correction) for about 18.5 per cent impurity at the end of the titration and 4.5 per cent other active impurity at the start yields a constant E'_0 value for the major portion of the titration curve.

TABLE 33.—*Titration of ethyl Capri blue nitrate with reduced anthraquinone-β-sulphonate in buffer No. 9, pH 4.901*

Reducing agent c. c.	E_h	E'_0	Reducing agent (*B*-corr.) c. c.	E'_0 (*B*-corr.)
2	0.1314	0.1027	1.1	(0.0979)
3	.1228	.1002	2.1	(.0987)
4	.1171	.0990	3.1	.0991
5	.1123	.0980	4.1	.0991
6	.1082	.0972	5.1	.0990
7	.1044	.0963	6.1	.0989
8	.1010	.0957	7.1	.0990
9	.0975	.0949	8.1	.0989
10	.0941	.0941	9.1	.0989
11	.0905	.0931	10.1	.0989
12	.0867	.0920	11.1	.0991
13	.0823	.0904	12.1	.0993
14	.0774	.0884	13.1	(.1001)
15	.0716	.0859	14.1	(.1028)
16	.0645	.0826	15.4	
17	.0561	.0787	Average = 0.0990	
18	.0455	.0742		
20				

Another reduction titration of the nitrate was made at pH 5.446 with new solutions. A smooth titration curve was obtained with a sharp endpoint. No precipitate was detectable in the colorless, reduced ethyl Capri blue. Allowing for 20 per cent "oxazone" at the end and 5 per cent other impurity at the start, there was obtained the average $E'_0 = 0.0487$.

The effect of high acidity on the formation of "oxazone" is well illustrated in the following titration at pH 1.995. The stock aqueous solution of ethyl Capri blue nitrate yielded only a slight coloration when shaken up with ether. On the other hand, the dye in buffer of pH 1.995 gave a marked coloration to the ether. Five cubic centi-

meters of the aqueous solution were added to 50 c. c. buffer No. 3, pH 1.995 and after thorough deoxygenation, titrated with reduced anthraquinone-β-sulphonate. The titration took 2½ hours to complete and an endpoint was obtained at 40 c. c. reducing agent. A plot of the potentials against cubic centimeters added shows two distinct plateaus corresponding to the titration of two distinct systems. Table 34, part A, gives the data for the Capri blue system. After correction for the equivalent of 0.7 c. c. active impurity at the start, there is obtained the average $E'_0 = 0.3608$. The potentials obtained in the latter part of the titration were somewhat erratic, the precision of measurement being ± 1 mv., but the evidence for the existence and interference of the presumed "oxazone" system is quite distinct in Table 34, part B.

TABLE 34.—*Titration of ethyl Capri blue nitrate with reduced anthraquinone-β-sulphonate in buffer No. 3, pH 1.995*

[PART A: CAPRI BLUE SYSTEM]

Reducing agent, c. c.	Reduction, per cent.	$0.03006 \times \log \frac{[S_r]}{[S_o]}$	E_h	E'_o	Reducing agent (*B*-corr.)	E'_o (*B*-corr.)
1	4.05	−0.0413	0.4110	0.3697	0.3	
2	8.10	−.0317	.3965	.3648	1.3	(0.3592)
3	12.15	−.0259	.3897	.3638	2.3	(.3604)
4	16.20	−.0215	.3849	.3634	3.3	.3609
5	20.25	−.0179	.3809	.3630	4.3	.3610
6	24.29	−.0149	.3775	.3626	5.3	.3610
7	28.34	−.0121	.3743	.3622	6.3	.3608
8	32.39	−.0096	.3716	.3620	7.3	.3608
9	36.44	−.0073	.3691	.3618	8.3	.3608
10	40.49	−.0050	.3667	.3617	9.3	.3607
11	44.54	−.0029	.3645	.3616	10.3	.3608
12	48.59	−.0007	.3623	.3616	11.3	.3608
13	52.64	+.0013	.3601	.3614	12.3	.3607
14	56.68	.0035	.3578	.3613	13.3	.3606
15	60.73	.0057	.3556	.3613	14.3	.3607
16	64.78	.0080	.3533	.3613	15.3	.3607
17	68.83	.0103	.3509	.3612	16.3	.3607
18	72.88	.0129	.3482	.3611	17.3	.3606
19	76.93	.0157	.3456	.3613	18.3	.3608
20	80.98	.0189	.3428	.3617	19.3	(.3612)
21	85.03	.0227	.3398	.3625	20.3	(.3620)
22	89.07	.0274	.3365	.3639	21.3	(.3635)
23	93.12	.0340	.3329	.3669	22.3	(.3665)
24.7	100.00				24.0	

Average = 0.3608

PART B: "OXAZONE" SYSTEM

Reducing agent, c. c.	Reduction, per cent	$0.0301 \times \log \frac{[S_r]}{[S_o]}$	E_h	E'_o
24.7	0.00			
26	8.50	−0.031	+0.320	+0.289
27	15.03	−.023	.315	.292
28	21.57	−.017	.310	.293
29	28.10	−.012	.303	.291
30	34.64	−.008	.296	.288
31	41.18	−.005	.290	.285
32	47.71	−.001	.285	.284
33	54.25	+.002	.281	.283
34	60.78	.006	.277	.283
35	67.32	.010	.274	.284
36	73.86	.014	.270	.284
37	80.39	.018	.261	.279
38	86.93	.025	.244	.269
39	93.46	.035	.218	.253
40	100.00			

Relation of E'_o to pH

The zinc chloride salt was used for this determination. A fixed mixture of reductant and oxidant was prepared and added to the various deoxygenated buffers to give a final concentration of 0.000025 M. The results are shown in Table 35 and in Figure 3. The potentials obtained in the different buffers pursued more or less characteristic courses. In general, the tendency was to drift negatively to a peak within the first 2 or 3 minutes and then to drift positively. The values chosen were these peak values, or failing this, the first obtainable measurement. Doubtful determinations were repeated. The measurements in the most alkaline buffers, Nos. 26 and 28, were rejected because immediate precipitation of dark blue material caused excessive drifts in electrode potentials.

The observed values of E'_o in table 35 show fair agreement with those calculated from the electrode equation.

Table 35.—*Ethyl Capri blue—Relation of E'_o to pH*

[Total concentration 0.000025 molar]

Buffer No.	pH	E'_o observed	E'_o calculated [1]	Deviation
1	1.076	+0.443	+0.443	.000
2	1.363	.415	.417	−.002
3	1.995	.359	.360	−.001
4	2.454	.318	.319	−.001
5	2.879	.280	.280	.000
6	3.329	.240	.240	.000
7	3.853	.193	.193	.000
8	4.419	.142	.142	.000
9	4.898	.099	.099	.000
10	5.397	.054	.054	.000
13	5.870	+.012	+.013	−.001
14	6.258	−.020	−.020	.000
14½	6.515	−.040	−.040	.000
15	6.731	−.054	−.055	+.001
15½	7.052	−.073	−.075	+.002
16	7.426	−.091	−.093	+.002
17	7.789	−.108	−.107	−.001
20	8.038	−.115	−.116	+.001
21	8.382	−.128	−.127	−.001
22	8.649	−.134	−.136	+.002
23	9.248	−.154	−.154	.000
23½	9.584	−.162	−.164	+.002
24	10.10	−.181	−.180	−.001

[1] Calculated with equation (14) using

$E'_{pH\ o}=0.540$ $K_{r2}=7.245\times10^{-6}$

$pK_{r2}=7.14$ $K_{r3}=1.995\times10^{-7}$

$pK_{r3}=6.70$

Electrode Equation and Dissociations

The electrode equation for the ethyl Capri blue system is obviously the same as that for the methyl homologue.

$$E_h=E_o-0.03006\log\frac{[S_r]}{[S_o]}+0.03006\log(K_{r2}K_{r3}[H^+]+K_{r3}[H^+]^2+[H^+]^3) \quad . \ . \ . \quad (14)$$

The dissociation constants were determined by graphical analysis of the E'_o:pH curve (Figure 3). No dissociation of the oxidant occurs in the pH range investigated. That is, K_{ob} is too great to be measured and the oxidant is completely dissociated in this range.

There are two bends in the E'_0:pH curve, and their directions indicate dissociations in the reductant, leuco-ethyl Capri blue. The values of the corresponding acid dissociation constants are estimated graphically as:

$$pK_{r2}=7.14; \quad K_{r2}=7.25\times10^{-8};$$
$$pK_{r3}=6.70; \quad K_{r3}=2.00\times10^{-7}.$$

General Discussion

The foregoing study has accomplished its purpose in so far as it has established the general outlines of the oxidation-reduction characteristics of the oxazine group of dyes. On the scale of electrode potential, they cover the range between methylene blue and indigo carmine (see fig. 3). The phenoxazine systems, methyl Capri blue, ethyl Capri blue, and cresyl blue are more electropositive than the naphthoxazine Nile blue in dilute solution. The latter system in more concentrated solution, between pH 1 and 7, is shifted to more electronegative values. There is given in Table 36 a survey of the potentials (E'_0 values) of the oxazines and of the other oxidation-reduction systems lying in the same range on the potential scale. They are arranged in the order of increasing E'_0 at pH 7.0. This tabulation should be useful to the investigator seeking available oxidation-reduction indicators in the range covered. It will be noted that basic compounds are now available where previously only the acid indigo sulphonates could be employed. This is a definite advantage when specific peculiarities of the acid indicators make it necessary to resort to basic compounds, and vice versa.

TABLE 36.—*E'_0 values at 30° C. of the oxazines and of other oxidation-reduction indicators lying within the same range of potential (values rounded to nearest millivolt and arranged in order of increasing potential at pH 7.0)*

pH	Indigo disulphonate	Nile blue (dilute) [1]	Indigo trisulphonate	Ethyl Capri blue	Methyl Capri blue	Indigo tetrasulphonate	Methylene blue	Cresyl blue
5.0	−0.010	−0.011	0.032	0.089	0.038	0.065	0.101	0.149
5.2	−.022	−.023	.020	.072	.025	.053	.088	.136
5.4	−.034	−.035	+.008	.054	+.012	.041	.077	.123
5.6	−.045	−.047	−.004	.036	−.000	.029	.066	.111
5.8	−.057	−.059	−.016	.019	−.011	.017	.056	.100
6.0	−.069	−.071	−.028	+.001	−.021	+.006	.047	.089
6.2	−.081	−.082	−.039	−.015	−.031	−.006	.039	.080
6.4	−.092	−.093	−.051	−.031	−.039	−.017	.031	.070
6.6	−.104	−.103	−.061	−.046	−.047	−.027	.024	.062
6.8	−.114	−.113	−.072	−.060	−.054	−.037	.017	.054
7.0	*−.125*	*−.122*	*−.081*	*−.072*	*−.061*	*−.046*	*.011*	*.047*
7.2	−.134	−.130	−.091	−.083	−.068	−.055	+.004	.040
7.4	−.143	−.138	−.099	−.092	−.074	−.062	−.002	.034
7.6	−.152	−.145	−.107	−.100	−.080	−.070	−.008	.027
7.8	−.160	−.152	−.114	−.108	−.086	−.077	−.014	.021
8.0	−.167	−.159	−.121	−.115	−.093	−.083	−.020	.015
8.2	−.174	−.165	−.127	−.121	−.099	−.090	−.026	.009
8.4	−.180	−.172	−.134	−.128	−.105	−.096	−.032	+.002
8.6	−.187	−.178	−.140	−.134	−.111	−.102	−.038	−.004
8.8	−.193	−.185	−.146	−.140	−.117	−.108	−.044	−.010
9.0	−.199	−.192	−.152	−.146	−.123	−.114	−.050	−.016

[1] 0.000025 molar.

Such a condition was encountered by Chambers, Pollack, and Cohen (1929) in their studies on the reduction potentials of marine ova. These cells were able to reduce the electropositive indicator systems down to and including methylene blue. The next compound in the series was indigo tetrasulphonate, and it apparently was not reduced by the cellular cytoplasm. However, ethyl Capri blue nitrate [8] was tried and evidence was obtained that the ova partially reduced this indicator under aerobic conditions. Since the basic Capri blue system is somewhat more electronegative than indigo tetrasulphonate, the inference was made that a peculiarity (possibly toxicity) of the acid dye was responsible for the failure of the cell to reduce it.

Some caution must be exercised in the use of the oxazines as indicators because of their tendency to decompose in aqueous solution. This tendency seems to be of about the same type and degree as occurs with methylene blue.

The concentration of the dyes is also of importance. Nile blue is a special case wherein the colloidal properties of the compound accentuate the concentration effect which was encountered to a much lesser degree in the other oxazines and in methylene blue. The low solubility of Nile blue and the great ease with which it is salted out of solution make it undesirable as a useful indicator of oxidation-reduction. On the other hand, it is precisely this property which renders Nile blue so useful as a stain and vital marker in cytological technique.

The concentration effect of cresyl blue is negligibly small with solutions of the order of 0.0001 molar provided the ionic strength of the buffer salts is held down to 0.2 μ or less. Methyl Capri blue is much like its thiazine analogue, methylene blue, in exhibiting a small but definite concentration effect even at the low concentration of 0.00002 molar. The presence of salts increases this effect appreciably. The same applies to ethyl Capri blue.

Both ethyl and methyl Capri blue also resemble methylene blue in that their leuco-compounds are light-sensitive and undergo oxidation on exposure to light.

On the effects of substitution.—Replacement of the sulphur atom of methylene blue by an oxygen atom causes a shift of the resulting methyl Capri blue system electronegatively. The difference between $E'_{pH\,0}$ values, so-called normal potentials, for the two systems ($0.477 - 0.532 = -0.055$) gives the difference in their free energy levels at the point $pH = 0$; but if this method of comparison be applied to ethyl Capri blue and methylene blue the result ($0.540 - 0.532 = +$

[8] For certain technical reasons the zinc chloride double salt was used in the electrometric calibration of some of the dyes. Obviously, for biological experiments the toxic zinc must be replaced by the more compatible nitrate or chloride.

0.008) is inconsistent and lacks significance. The result is obviously influenced by the values of the several dissociation constants of these basic compounds. The other alternative of a comparison of the energies of reduction of the free bases is not feasible because the dissociations of the strong polar groups are as yet beyond confident experimental reach. Rough experiments indicate that Nile blue base can be sublimed. If the free bases of the other compounds can also be isolated and sublimed, it may be that the free energy changes of the reversible reductions referred to the gaseous state will provide as satisfactory a reference state for these basic compounds as Conant (1927) found for the quinones.

Now, preliminary measurements show that the diazine and safranine systems are more electronegative than the oxazines. Apparently, there occurs a progressive advance in electronegativity of the corresponding oxidation-reduction systems as one of the bridging positions (in the type structure under consideration) is occupied by sulphur (in thiazines), by oxygen (in oxazines), and by nitrogen (in diazines). It will be noted that there is a regular progression in mass and electronegativity of the substituting atoms. It would seem, therefore, that the substitution of carbon in the same bridging position (as in the carbazines) should be accompanied by a still more electronegative position of the corresponding oxidation-reduction system on the scale of electrode potential. This prediction will be tested experimentally.

TABLE 37.—*Comparison of Oxazines and Thiazines—Basic ionization constants, pH values of bends in E'_o : pH curves (corresponding to pK_a values) and characteristic potentials at pH=0*

Group	Symbol	Methyl Capri blue		Ethyl Capri blue		Cresyl blue	
		Value of constant	Bend at pH	Value of constant	Bend at pH	Value of constant	Bend at pH
Oxidant's polar	K_{ob}	Too high to measure	None.	Too high to measure	None.	1.0×10^{-3}	10.7
Oxidant's nonpolar	$K_{ob2}(K_{o2})^1$	Too low to measure	None.	Too low to measure	None.	Too low to measure	None.
Reductant's bridging N	$K_{rb1}(K_{r1})^1$	Fixes H+	None.	Fixes H+	None.	Fixes H+	None.
Reductant's 1st basic	$K_{rb2}(K_{r2})^1$	2.37×10^{-8}	6.10	2.60×10^{-7}	7.14	3.76×10^{-8}	6.3
Reductant's 2nd basic	$K_{rb3}(K_{r3})^1$	1.33×10^{-9}	4.85	9.44×10^{-8}	6.70	7.50×10^{-10}	4.6
Characteristic potential	$E'_{pH\ 0}$	0.477		0.540		0.583	

Group	Symbol	Lauth's violet		Methylene blue		Nile blue (dil. sol.)	
		Value of constant	Bend at pH	Value of constant	Bend at pH	Value of constant	Bend at pH
Oxidant's polar	K_{ob}	1.88×10^{-3}	11.0	Too high to measure	None.	9.94×10^{-3}	9.7
Oxidant's nonpolar	$K_{ob2}(K_{o2})^1$	Too low to measure	None.	Too low to measure	None.	Too low to measure	None.
Reductant's bridging N	$K_{rb1}(K_{r1})^1$	Fixes H+	None.	Fixes H+	None.	Fixes H+	None.
Reductant's 1st basic	$K_{rb2}(K_{r2})^1$	3.8×10^{-9}	5.30	1.35×10^{-8}	5.85	1.50×10^{-7}	6.90
Reductant's 2nd basic	$K_{rb3}(K_{r3})^1$	4.5×10^{-10}	4.38	6.3×10^{-10}	4.52	1.57×10^{-10}	3.92
Characteristic potential	$E'_{pH\ 0}$	0.563		0.532		0.406	

[1] $K_{o1}=\frac{K_w}{K_{ob2}}$, etc.

A comparison of the basic dissociations reveals the close similarity of the oxazines and the thiazines. The data are collected in Table 37 to facilitate inspection. Comparing methylene blue and methyl Capri blue, it will be noted that the orders of magnitude of the various dissociation constants are the same so far as can be determined. A detail which may be of significance is that the basic constants of the oxazine reductant are somewhat greater than those of the analogous thiazine reductant. Noteworthy also is the maintenance in these complex organic systems of the general rule that the magnitude of the dissociation of a substituted amine increases in the order $NH_2 < N(CH_3)_2 < N(C_2H_5)_2$. A more extensive series of homologous compounds would have to be examined to permit an analysis of the numerical energy relations involved.

It is necessary to consider for a moment a determination of the basic dissociation of cresyl blue reported by Irwin (1926). This was obtained by measurements of the partition of dye between chloroform and aqueous buffer solutions. The value thus found was $10^{-5.6}$ (a relatively weak base) as compared with 10^{-3} (a stronger base) determined from the E'_0: pH curve. The discrepancy is far beyond the experimental error and the two values must refer to two distinct systems.

Since dissociation constants as determined experimentally are apparent constants which include the effects of tautomerization, etc., it is hardly permissible to claim that the two divergent values correspond to different tautomers of the same compound. The identity of the weak base with $K_b = 10^{-5.6}$ is not established, but experiment *suggests* that it is a breakdown product intermediate between cresyl blue and its oxazone. Fresh cresyl blue solutions in water give a blue color at pH 7, 8, 9, and 10, and a nonfluorescent brown red at pH 12 and 13. The dissociation near pH 11 corresponds to that of the relatively strong base of cresyl blue, $K_b = 10^{-3}$. If these solutions be allowed to stand for several days one notes a secondary color change. The solution at pH 7 still seems clear blue but at pH 9 and 10 the original blue has given way to a pale fluorescent violet-pink easily extractable with benzene.

In the formulation of the oxidation-reduction data and the evaluation of the various constants the simplest general relations have been assumed. The pronounced colloidal nature of Nile blue and the less serious concentration effects of the other compounds make it at once evident that a complete and thorough elucidation of their behavior in solution must include the application of the activity concept. The present investigation has merely revealed the existence of the phenomena as they interfered with the general problem.

On the micelle formation of Nile blue.—The conclusion that aggregation of Nile blue molecules is due to the polar ion, Ox^+, seems a logical deduction from the experimental evidence although the data

are not complete in every detail. The concentration effect on the equilibrium potentials and on the spectral absorptions support the view that aggregation takes place. The observation that the concentration effect is reversed in alkaline regions as the pH of the medium approaches that at which dissociation of Ox^+ is suppressed seems convincing evidence that it is the dissociated polar oxidant which undergoes aggregation.

While no analytical comparison of the data for "dilute" and "concentrated" solutions has been given here, it may be stated that the uncertainty of the data in the alkaline region is due to the low solubility of the free base of Nile blue. Making allowance for this, it appears that at pH 11 and higher the E'_0: pH curves for dilute and concentrated solutions superpose each other; that is, the effect of micelle formation is absent when dissociation of polar oxidant is suppressed.

On the formation of oxazone.—Thorpe (1907) made a study of the hydrolytic oxidation of Nile blue to the corresponding oxazone and established the identity of the latter. Heating an acidified solution of Nile blue will convert it in a short time almost entirely into the fluorescent red product which is soluble in xylene; the parent substance is insoluble in this solvent. According to Thorpe, this property is confined to the naphthoxazines, because under the same conditions certain phenoxazines gave no trace of oxazone. Only after heating acidified solutions of Capri blue GN and GON for several days in the presence of xylene did he obtain a faint red coloration. This statement in reference to the phenoxazines has not been confirmed in the present study. On the contrary, the constant occurrence of a xylene-soluble red product in aqueous solutions of the phenoxazines, cresyl blue and the Capri blues, seems practically unavoidable, as the foregoing experiments definitely show.

The following observations suggest an explanation for the difference found by Thorpe in the formation of naphthoxazones and phenoxazones. The oxazone of Nile blue is an extremely weak base, and when suspended in 5 per cent HCl and shaken with xylene gives an intense fluorescent red coloration to the xylene while the aqueous phase is practically colorless. Neutralization of the acid produces no change in the above extraction. On the other hand, the oxazones of cresyl blue and Capri blue are stronger bases than Nile blue oxazone and under the same conditions are retained in the aqueous acid layer as salts while the xylene layer has practically no coloration. When the acid is neutralized with KOH, the aqueous layer loses nearly all its color and the xylene becomes colored fluorescent red.

It therefore follows that boiling phenoxazines such as these with dilute acid in the presence of xylene should give no coloration to the latter until the salt of the oxazone formed is converted to the base which can be taken up by the solvent.

Acknowledgments.—For most of the chemical analyses reported in this paper, we are indebted to Assistant Chemist, C. G. Remsburg, National Institute of Health. We wish also to express our appreciation to W. C. Holmes, Bureau of Chemistry, for the recrystallized cresyl blue and other compounds generously offered; to Doctor Appel, Bureau of Standards, for making available samples from the Textile Alliance collection of dyes; and to Miss M. E. Perkins, Department of Physiological Chemistry, Johns Hopkins School of Medicine, for assistance in the spectrophotometric work and in the arduous computations and checking of the final data.

Summary and Conclusions

The equilibrium potentials of the oxazines: Nile blue A, brilliant cresyl blue, methyl Capri blue and ethyl Capri blue, were determined at 30° C. in buffers of various pH values.

For the Nile blue system in dilute solution and for the cresyl blue system, the rationally developed electrode equation found to be applicable is

$$E_h = E_o - \frac{RT}{2F} ln \left[\frac{[S_r]}{[S_o]} \times \frac{(K_{ob}[H^+] + K_w)}{K_{r2}K_{r3}[H^+]^2 + K_{r3}[H^+]^3 + [H^+]^4} \right].$$

For the Capri blue systems this equation is modified to

$$E_h = E_o - \frac{RT}{2F} ln \left[\frac{[S_r]}{[S_o]} \times \frac{1}{K_{r2}K_{r3}[H^+] + K_{r3}[H^+]^2 + [H^+]^3} \right].$$

In these equations, E_h is the potential difference between electrode and solution; E_o is a characteristic constant defined by the relation $E_o = E'_{pH\ 0} + \frac{RT}{2F} ln\ K_{ob}$; $[S_o]$ and $[S_r]$ are the concentrations of total oxidant and total reductant, respectively. The several dissociation constants for each system are defined as follows:

K_{ob} = dissociation constant of the polar amino group of the oxidant,
K_{r2} = dissociation constant of the hydrion from the first amino group of the reductant,
K_{r3} = dissociation constant of the hydrion from the second amino group of the reductant,
K_w = dissociation product of water.

One hydrogen atom in the reductant is considered fixed to the bridging nitrogen.

TABLE 38.—*Values of the constants of the oxazine systems*

Constant	Methyl Capri blue		Ethyl Capri blue		Cresyl blue		Nile blue (dil. sol.)	
	K_{acid}	Corresponding to basic constant	K_{acid}	Corresponding to basic constant	K_{acid}	Corresponding to basic constant	K_{acid}	Corresponding to basic constant
K_{ob}	----------	Very high.	----------	Very high.	----------	1.0×10^{-3}	----------	9.94×10^{-5}
K_{ob2}	----------	Very low.	----------	Very low.	----------	Very low.	----------	Very low.
K_{r1}	----------	Very low.	----------	Very low.	----------	Very low.	----------	Very low.
K_{r2}	7.94×10^{-7}	2.37×10^{-8}	7.25×10^{-8}	2.6×10^{-7}	5.01×10^{-7}	3.76×10^{-8}	1.26×10^{-7}	1.5×10^{-7}
K_{r3}	1.41×10^{-3}	1.33×10^{-9}	2.0×10^{-7}	9.44×10^{-8}	2.51×10^{-5}	7.5×10^{-10}	1.2×10^{-4}	1.57×10^{-10}
K_w	1.88×10^{-14}	----------	----------	----------	----------	----------	----------	----------
E'_{pHO}	0.477	----------	0.540	----------	0.583	----------	0.406	----------

In methyl and ethyl Capri blue the polar substituted amino group of each oxidant is so strongly basic that its salt is completely dissociated throughout the pH range studied. In cresyl blue, the disso-

ciation of the polar oxidant is detectable at pH 10.7. It is a somewhat stronger base than ammonia. In Nile blue (dilute solution) the polar oxidant dissociates near pH 9.7 and has about the same "strength" as ammonium. The values of the various constants were determined graphically from the E'_o:pH curves (Fig. 3) and are given in Table 38.

The analyses and chemical properties of Nile blue sulphate indicate the constitution of this salt to be $C_{20}H_{20}N_3O \cdot HSO_4$ and not the dibasic salt $(C_{20}H_{20}N_3O)_2SO_4$ which is currently accepted.

The colloidal state of aqueous solutions of Nile blue imposes marked salt and concentration effects on the electrode potentials. In acid solutions the relation between concentration, C, and potential, E, is described by the equation

$$C = ae^{-bE}$$

The values of the constants a and b depend on the pH and the ionic strength. Not until the dye concentration is reduced to the order of millionth molar does a Nile blue solution begin to behave like a "true" dilute solution. The spectrophotometric and electrometric measurements suggest that the polar oxidant of Nile blue in water aggregates to micelles of a complexity depending on dye concentration and on the thermodynamic environment. Micelle formation appears to be reversed in the alkaline region where dissociation of the polar dialkyl-amino group of the oxidant is suppressed by hydroxyl ion concentration. Aggregation is also reversed by dilution.

Relatively small but definite concentration and salt effects occur with cresyl blue, methyl Capri blue, and ethyl Capri blue.

A notable characteristic of the oxazines is their ready reactivity with water to form oxazones and intermediate products of oxidative hydrolysis. In this they strongly resemble methylene blue. Strictly speaking, therefore, these compounds remain pure only if kept out of contact with water. Moderate alkalinity and acidity appear to accelerate such decompositions.

Until more stable compounds are found the phenoxazines, cresyl blue, methyl Capri blue and ethyl Capri blue can serve as indicators of oxidation-reduction within the range of electrode potential bounded by methylene blue and indigo carmine. Their basic properties may make them desirable in certain cases when the acid indigo sulphonates can not be employed.

Nile blue A is the most electronegative of the systems studied, and its E'_o:pH curve almost superposes that of indigo carmine. The poor solubility, colloidal nature and marked concentration effect of this dye impair its utility as an oxidation-reduction indicator but make it admirably suited as experimental material for the study of colloidal phenomena.

Appendix

After the termination of the electrode measurements reported in the foregoing study, there appeared the papers by Rapkine, Struyk, and Wurmser (1929) and Vellinger (1929) in which the oxidation-reduction potentials of Nile blue and cresyl blue are reported. In neither paper are the actual numerical values given; instead, the data are presented along curves on a small-scale chart.

The first group of investigators determined the potentials by titrations with titanous chloride at 18°. Their E'_0: pH curves cover the range of pH 4 to 9 for cresyl blue and pH 5 to 9 for Nile blue. These curves give a general idea of the positions of these systems on the potential scale but details for even a rough physico-chemical characterization are absent. It should also be noted that Rapkine, Struyk, and Wurmser's cresyl blue is stated to be the diethyl homologue.

Vellinger employed $Na_2S_2O_4$ as a titrating agent; the temperature is not given. His E'_0: pH curves cover the range of pH 1 to 12 for cresyl blue and pH 4 to 8 for Nile blue. The Nile blue curve does not show the bend we find near pH 7. His cresyl blue curve does not show the 0.06 slope between pH 4 and 6 nor the 0.03 slope in the extreme alkaline zone. From pH 6 to 12 his values for the cresyl blue system agree fairly well with those reported in our paper, but in the acid region the divergence is rather large, perhaps because of higher dye concentration. However, in the absence of more definite data it is hard to decide if Vellinger was working with dimethyl cresyl blue or with a different homologue.

The following tabulation brings the data together for inspection. They are not strictly comparable for obvious reasons. It should be emphasized, therefore, that the purpose in juxtaposing them is to place before the reader the available information on the subject.

	Nile blue				Cresyl blue		
Authors	Cohen and Preisler		Rapkine, Struyk, Wurmser	Vellinger	Cohen and Preisler	Rapkine, Struyk, Wurmser	Vellinger
Temperature	30°		18°	(?)	30°	18°	(?)
Concentration (M)	0.000136	0.000025	0.00016 to 0.00026	0.00027	0.000025	0.00039	0.00034
pH	E'_0	E'_0	E'_0	E'_0	E'_0	E'_0	E'_0
1.0					+0.522		+0.355
2.0					.402		.300
3.0					.312		.245
4.0	+0.042	+0.056	+0.059	+0.070	.225	0.210	.190
5.0	−.021	−.011	−.027	−.025	.149	.138	.135
6.0	−.081	−.071	−.085	−.090	.089	.067	.080
7.0	−.116	−.122	−.143	−.150	.047	+.023	.045
8.0	−.153	−.159		−.205	+.015	−.010	+.020
9.0	−.179	−.192			−.016	−.043	−.015
10.0					−.022		−.035
11.0					−.054		−.070
12.0					−.095		−.095

NOTE.—The values cited from the work of R. S. and W. and of V. were read from their published charts.

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